











NATIONAL INSTITUTE OF  
DIABETES AND DIGESTIVE  
AND KIDNEY DISEASES

ANNUAL REPORTS

DIVISION OF INTRAMURAL  
RESEARCH

October 1, 1989 to September 30, 1990

LIBRARY

SEP 10 1991

National Institutes of Health

RC  
620  
N 279  
1990

ANNUAL REPORTS

DIVISION OF INDIAN AFFAIRS  
RESEARCH

1989 to September 30, 1990

## PREFACE

As the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) begins the celebration of its 40th Anniversary, we are pleased to present this Annual Report covering the activities and achievements of our Division of Intramural Research during the year just ended. Since its establishment as a separate institute at NIH four decades ago, the NIDDK has been responsible for research on a broad spectrum of diseases affecting virtually every family in the Nation. These diseases are among the most prevalent, chronic, disabling and costly that we face; they afflict millions of Americans of all ages, backgrounds and economic circumstances and constitute an enormous drain in terms of human suffering and economic costs. Their economic impact alone exceeds \$75 billion annually.

Despite the apparent diversity marking the broad array of diseases within NIDDK's purview, these disorders share, to a great extent, mutual scientific and biomedical denominators. In many of them the common threads of molecular and cellular biology, endocrinology, metabolism, immunology and nutrition run throughout. Thus, quite often, new knowledge generated in one group, leads to progress against the others. Though, externally the NIDDK appears to serve multiple interests, internally, its programs are intertwined and benefit from a close, synergistic relationship.

All of our studies have the goal of developing new knowledge that will ultimately permit us to treat, control, or prevent the diseases within our domain of responsibility. Such new knowledge must begin at the cellular and subcellular level with major emphasis on basic studies and become extended, where promising, to clinical investigations. We are justly proud of the excellent record of achievement of NIDDK's intramural research program in this long and arduous process, and we are sure that this compendium will convey to the reader a spirit of creativity and responsiveness to challenge.

This report would be incomplete without warm acknowledgement of the tireless work, dedication and talent of our intramural scientists who have conducted the studies presented here. Our hopes for progress in the Nineties and beyond rest squarely on their shoulders.

Phillip Gorden, M.D.  
Director  
National Institute of  
Diabetes and Digestive and Kidney Diseases



# TABLE OF CONTENTS

## PREFACE

Dr. Phillip Gorden, Director .....	i
------------------------------------	---

## DIVISION OF INTRAMURAL RESEARCH

Dr. Jesse Roth, Director	
Dr. Edward Steers, Acting Associate Director	
Dr. N. Raphael Shulman, Acting Deputy Director	

## PROJECT REPORTS

### MATHEMATICAL RESEARCH BRANCH

Summary .....	1
Mathematical formulations and analysis relevant to experimental neurophysiology .....	9
Mathematical description of substrate transport in capillary-tissue structures .....	10
Mathematical description of cellular neuroelectric signal transmission .....	11
Probabilistic analyses of nucleic acid sequences .....	12
Sound processing in the auditory system .....	13
Electrical and chemical oscillations in coupled cell systems .....	14

### LABORATORY OF CELLULAR AND DEVELOPMENT BIOLOGY

Summary .....	15
Modulation of hormone responsive system by RAS oncogene product .....	26
Regulation of adipocyte metabolism .....	27
Protein nucleic acid interactions: chromatin structure and function .....	28
Study of ribonuclease and its inhibitor from bacillus amyloliquefaciens .....	29
Studies of folic acid (dihydrofolate reductase) .....	30
Hormones, lipoprotein lipase and lipid metabolism .....	31
Synthesis and transport of lipoprotein and hepatic lipases in tissues and cells .....	32
Ultrastructural immunocytochemistry of lipid metabolism in cultured cells and tissues .....	33
Large-scale processing of biological material .....	34
Regulation of developmental gene expression .....	35
Control of gene expression in early mammalian development .....	36
Chromatin structure in regulation of mammalian gene expression .....	37

## LABORATORY OF BIOCHEMISTRY AND METABOLISM

Summary .....	38
The role of the carbohydrate moiety of glycoprotein in cellular activity .....	47
Enzymatic basis of detoxication .....	48
Polysaccharides in morphogenesis .....	49
Thermodynamic and kinetic studies of protein structure and enzymic mechanisms .....	50
The role of the nuclear envelope in intracellular protein sorting .....	51
Tissue specific and hormone regulated gene expression .....	52
The genetic lesions of Tay-Sachs disease .....	53
Electrochemical ion gradients as a mechanism of cellular message transmission .....	54
Cell regulation by pharmacodynamic and autoimmune agents acting on cell membranes .....	55
Endocytosis, secretion and compartmentalization in mutant CHO cells .....	56
The role of intracellular traffic in HIV infection .....	57
Cell specific activity of elements within the HIV- LTR .....	58
Direct measurement of forces between membranes or macromolecules .....	59
Physics of ionic channels and other proteins with aqueous cavities .....	60
Structure and physical properties of DNA and DNA - protein complexes .....	61
Histamine release on a hydration of Granule matrices .....	62
Cell-cell fusion due to influenza hemagglutinin .....	63

## LABORATORY OF CHEMISTRY

Summary .....	64
Reactions and immunochemistry of carbohydrates .....	77
Analogues of thyrotropin - releasing hormone .....	78
Stereopopulation control in drug delivery and enzyme simulation .....	79
Chemistry of bioimidazoles .....	80
Halogenated biogenic amines in biochemistry and pharmacology .....	81
Significance of ligand tautomerism in biorecognition .....	82
Functionalized congeners of bioactive compounds .....	83
Prosthetic groups for radiolabeling of functionalized drugs and peptides .....	84
Development of drugs acting at adenosine receptors .....	85
Aldose reductase inhibitors .....	86
Novel amino acids for conformational and stereochemical constraints in peptides .....	87
Fluorinated analogue of bioactive peptides .....	88

## LABORATORY OF CELL BIOLOGY AND GENETICS

Summary .....	89
Cytogenetics .....	107
Mechanisms of hormone and transmitter secretion .....	108

## LABORATORY OF BIOCHEMICAL PHARMACOLOGY

Summary .....	109
Biochemistry of sulfur-containing compounds .....	122
Aldoheptose biosynthesis and its regulation and hepatitis non-A, non-B .....	123
Mammalian transposons .....	124
Bacteriophage T4 gene expression .....	125
Structure and function of the tryptophan synthase multienzyme complex .....	126
Noncovalent: intermolecular interactions in biochemistry .....	127
Enzymatic mechanisms of DNA replication: The bacteriophage T4 system .....	128
Structure and interactions of biologically important macromolecules .....	129
Polyamine biosynthesis and function .....	130
Yeast RNA virology .....	131

## LABORATORY OF CHEMICAL BIOLOGY

Summary .....	132
The core loop interaction that controls protein folding .....	134
The core interaction loops and core loop coalescence energy in protein folding .....	135
Trans-acting factor(s) controlling globin gene expression in K562 cells .....	136
Sickle cell anemia: The intracellular polymerization of hemoglobin S .....	137
The origin of specificity of antigen-antibody interaction .....	138
The development of non-invasive methods to assess sickle cell patients .....	139
Effects of HTLV-I Tat-I product of globin gene expression .....	140
Regulation of beta globin gene expression .....	141
Regulation of human T cell receptor delta and alpha gene usage .....	142
Function, ligand, and ontogeny of expression of the gamma, delta T cell receptor .....	143
Laboratory model of adult globin gene expression .....	144
Trans-activating factors and globin gene expression: A direct approach .....	145

In vitro transcription of human globin genes with K562 nuclear extracts .....	146
Isolation of embryonic globin transcriptional factors by subtractive cDNA cloning .....	147
Effect of hydroxyurea on fetal hemoglobin synthesis in sickle cell patients .....	148
Cytogenetic investigations of patients with genetically determined disorders .....	149
Aids: transcriptional regulation by the TAT-protein LTR of HIV in vitro .....	150
Regulation of globin gene expression by upstream positive control DNA sequences .....	151
Coordinated expression of human beta sickle antilles and human alpha globin in transgenic mice .....	152
Analysis of the Epsilon Globin gene flanking sequences .....	153
Trans-regulation of human globin genes .....	154
Control of transcription of erythropoietin .....	155
The erythropoietin receptor and genetic control red cell development .....	156
Mechanism(s) of enhanced gamma globin gene expression in patients .....	157
Production and Characterization of HIVTAT .....	158
Trans-acting factor(s) controlling Epsilon globin gene expression .....	159
$\beta$ -Globin gene expression in patients with different type of $\beta$ -thalassemia mutation .....	160

#### LABORATORY OF CHEMICAL PHYSICS

Summary .....	161
Molecular dynamics and vibrational characteristics of membrane assemblies .....	166
Chemistry of natural compounds, and synthetic organic chemistry .....	167
Asymmetric synthesis: Structure, stereochemistry, and NMR .....	168
The structure and dynamic properties of macromolecules .....	169
Structure and interaction of biomolecules .....	170
Electric and molecular structural investigation .....	171
Studies on sickle cell disease .....	172
Dynamics of proteins and studies on sickle cell disease .....	173
The physics and chemistry of photoreception .....	174
Macromolecular dynamics and assembly reactions .....	175
Spectroscopic investigation of membrane lipids and models .....	176
Theoretical studies on the dynamic aspects of macromolecular function .....	177
Nuclear magnetic resonance: New methods and molecular structure determination .....	178



Conformation and dynamics of biological macromolecules .....	179
Structural studies of AIDS proteins by NMR .....	180
Determination of three-dimensional structures of macromolecules in solution by NMR .....	181
Investigations of macromolecular structures and dynamics solution by NMR .....	182
NMR and other spectroscopic studies of molecular structure .....	183
Theoretical studies of dynamical processes in chemical physics and biophysics .....	184
 LABORATORY OF BIOORGANIC CHEMISTRY	
Summary .....	185
Pharmacologically active compounds from amphibians and other natural sources .....	198
Pharmacology and metabolism of biogenic amines and related compounds .....	199
Ion channels receptors and second messengers in the nervous system .....	200
Enzymatic oxidation of drugs to toxic and carcinogenic metabolites .....	201
Nicotinic and muscarinic acetylcholine receptor agonists .....	202
Mechanistic enzymology of HIV proteins .....	203
Mass spectrometry of drugs, metabolites and natural products .....	204
Adenosine receptor agonists and antagonists .....	205
Interaction between second messengers systems .....	206
 LABORATORY OF MOLECULAR BIOLOGY	
Summary .....	207
Studies of functions involved in genetic recombination .....	216
Studies of immunoglobulin gene rearrangement .....	217
Effects of DNA supercoiling on the topological properties of nucleosomes .....	218
Studies on mechanism of genetic recombination .....	219
Chromatin structure and function .....	220
Enzyme structure .....	221
Three-dimensional structure of proteins of the immune system .....	222
Chemical and structural investigations of nucleic acids and related molecules .....	223
Replication, recombination, and repair of microbial DNA .....	224
Nonheritable antibiotic resistance .....	225
Mammalian DNA replication, regulation and amplification .....	226
Energy conversion in biology .....	227
Statistical thermodynamics of protein and polynucleotide systems .....	228

Thermal measurements of biomolecular systems .....	229
Influences of macromolecular crowding on biochemical systems .....	230
Developmental regulation of differential gene expression .....	231
Studies on the mechanism of retroviral DNA integration .....	232
AIDS related proteins: Structure and function .....	233
Control of gene expression during chicken erythrocyte development .....	234
Structural molecular biology .....	235
Regulation of a gene expressed in undifferentiated teratocarcinoma cells .....	236
Channeling in the biosynthesis of histidine .....	237

#### METABOLIC DISEASES BRANCH

Summary .....	238
Structure, secretion, and mechanism of action of parathyroid hormone .....	248
Studies on the mode of action of thyrocalcitonin .....	249
Study of hyperparathyroidism: Etiology, diagnosis, and treatment .....	250
Vitamin D resistance and related disorders .....	251
Regulation of mineral metabolism .....	252
Disorders of immune regulation in patients with systemic lupus erythematosus .....	253
Production and characterization of nephritic factor .....	254
Regulation of human immune response by complement .....	255
Immunosuppressive drug therapy in lupus glomerulonephritis .....	256
Renal biopsy pathology in systemic lupus erythematosus .....	257
Glomerular Disease Transgenic Mice .....	258
Histopathology of renal lesions in Pima Indians .....	259
Renal lesions in leukemias, lymphomas, and carcinomas .....	260
Regulation of expression of angiotensin converting enzyme in renal glomeruli .....	261
Biology of insulin receptors in glomerular cells .....	262
Pathogenesis of murine lupus nephritis .....	263
Membranes lupus nephropathy .....	264
Glomerular change due to GH and IGF-I .....	265
Effect of TGF- $\beta$ on glomerular cells .....	266
Role of IGF-I in biology of mouse glomerular cells .....	267
Biology of human glomerular mesangial cells .....	268
Binding and uptake of mouse IgA by mouse glomerular mesangial cells .....	269
Proteoglycan production by mouse glomerular epithelial cells .....	270
Idiopathic membranous nephropathy .....	271
Renal lesions in the ablation model: Role of growth factors .....	272
Identification of IGF-I binding proteins by mouse mesangial cells .....	273

Interactions between TGF-B and glomeruli .....	274
Renal lesions in non-obese diabetic mice .....	275
CLINICAL ENDOCRINOLOGY BRANCH	
Summary .....	276
Thyroxine-protein interactions .....	284
Structure of polypeptide and protein hormones .....	285
Studies in thyroid diseases .....	286
Membranes and secretion .....	287
Thyroid hormone secretion and the function of microtubules .....	288
Adenylate cyclase and other extracellular products of B pertussis .....	289
Thyroid hormones - cell interactions .....	290
Mapping of triiodothyronine responsive genes .....	291
Regulation of specific rat liver mRNAs by thyroid hormone .....	292
Molecular biology of thyroid hormone receptors .....	293
Effect of thyroid hormone on synthesis of myelin basic proteins .....	294
The role of POU - domain genes during <i>Xenopus laevis</i> embryogenesis .....	295
The role of XPO, a localized zinc finger gene, in anterior-posterior axis formation in <i>xenopus</i> .....	296
DIABETES BRANCH	
Summary .....	297
Phosphorylation of the insulin and IGF-I receptor .....	303
Insulin gene expression and insulin action .....	304
Studies of insulin receptors in circulation cells in man .....	305
Antibodies to receptors: Detection in disease states and use as probes .....	306
Positron emission tomography .....	307
Acromegaly and growth hormone .....	308
Cellular hormone-like peptides .....	309
Morphologic studies of ligand binding to cells .....	310
Insulin receptors in syndromes of extreme insulin resistance .....	311
Biosynthetic labeling of the insulin receptor .....	312
Tissue receptors for insulin and insulin-like growth factors .....	313
Tyrosine-specific protein kinase activity associated with the insulin receptor .....	314
Use of SMS 201-995 in hormone secreting tumors .....	315
Transcriptional regulation of the insulin receptor gene .....	316
CLINICAL HEMATOLOGY BRANCH	
Summary .....	317
Study of immunology of blood cell deficiencies .....	322
Study of blood coagulation and diseases of hemorrhage and thrombosis .....	323

## GENETICS AND BIOCHEMISTRY BRANCH

Summary .....	324
Gene expression and human genetics .....	327
Toxins and DNA repair in xenopus oocytes .....	328
Structure-function relationship of lysosomal enzymes .....	329
CD4 receptor structure/function project .....	330
Molecular studies of protein-DNA interactions .....	331

## DIGESTIVE DISEASES BRANCH

Summary .....	332
Studies of membrane function .....	343
Gastrointestinal hormones .....	344
Cyclic nucleotide mediated functions .....	345
Identification and characterization of Receptors for GI Peptides .....	346
Cellular basis of action of gastrointestinal peptides .....	347
Management of Islet cell tumors .....	348
Receptors on gastric smooth muscle cells .....	349
Studies relating to the pathogenesis of hepatic encephalopathy .....	350
Immunologic studies of primary biliary cirrhosis .....	351
Studies of alpha-1-antitrypsin phenotypes and metabolism .....	352
Studies of hepatic receptors for glycoproteins .....	353
Studies of the natural history and treatment of chronic type B hepatitis .....	354
Studies of natural history and treatment of chronic non-A, non-B, (type C) hepatitis .....	355
Trials of therapies for primary biliary cirrhosis .....	356
Immunological studies in chronic viral hepatitis .....	357
Studies of the natural history and treatment of Duck Hepatitis B virus infection .....	358
Studies of the opiate system in cholestatic liver disease .....	359

## MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH

Summary .....	360
Biosynthesis and glycosylation of thyrotropin .....	367
Regulation and action of thyrotropin .....	368
Molecular biology of pituitary glycoprotein hormones and hypothalamic releasing hormones .....	369
Insulin-like growth factors .....	370
Insulin-cell interaction .....	371
Insulin's regulation of glucose transport .....	372
Alterations in insulin's action in insulin-dependent diabetes mellitus .....	373
Insulin's regulation of hormone binding .....	374
Counterregulation of insulin's action by catecholamines .....	375

Alterations in insulin's action with fasting/refeeding .....	376
Mutations of the thyroid hormone receptor gene in patients with thyroid hormone resistance .....	378
LABORATORY OF STRUCTURAL BIOLOGY	
Summary .....	379
Biology of complex carbohydrates .....	381
Metabolism and role of polysaccharide sulfate .....	382
Expression and function of bacterial cell surface components in pathogenesis .....	383
LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY	
Summary .....	384
Function of DNA virus genomes in animal cells .....	387
Hormonal regulation of cell growth and differentiation .....	388
Studies on the metabolic defect in Sialuria .....	389
Regulation of HIV by AAV .....	390
LABORATORY OF ANALYTICAL CHEMISTRY	
Summary .....	391
Analytical services and methodology .....	396
Application of NMR in biochemical and biological systems .....	397
Initial intracellular events of steroid hormone action .....	398
The development of methods and materials for the study of medical problems .....	399
Professional practices of biomedical scientists .....	400
Interferon induction and action. The antiviral activity of nucleoside analogs .....	401
Chemistry and metabolism of Quinghaosu: A chinese antimalarial drug .....	402
Physostigmine and analogs .....	403
Mammalian alkaloids .....	404
Structure-Activity relationships of colchicinoids based on tubulin binding .....	405
Antiviral drugs .....	406
Beta-carbolines .....	407
Analogues of nucleic acids and their components as potential anti-AIDS agents .....	408
Oxindoles .....	409
Inhibition of vesicular stomatitis virus RNA polymerase by 2'5'-oligoadenylates .....	410
Nortropane alkaloids .....	411
Analytical reagents from dihydrofluorescein .....	412
LABORATORY OF NEUROSCIENCE	
Summary .....	413
Receptors for neurotransmitters and drugs in brain and peripheral tissues .....	417

## MOLECULAR PATHOPHYSIOLOGY BRANCH

Summary .....	418
Molecular biologic studies on the cause of parathyroid neoplasia .....	421
Guanine nucleotide binding proteins as receptor- effector couplers .....	422
Studies on pseudohypoparathyroidism and related disorders .....	423

## LABORATORY OF MEDICINAL CHEMISTRY

Summary .....	424
Design and synthesis of drugs acting on central and peripheral tissues .....	435
Design, synthesis and evaluation of medicinal agents and research tools .....	436

## PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH

Summary .....	437
Diabetes mellitus and other chronic diseases in the Gila River indian community .....	451
Complications and outcome of diabetic and prediabetic pregnancies .....	452
Muscle capillary basement membrane thickness prior to onset of diabetes .....	453
Gila River indian community autopsy and mortality study .....	454
Natural history of arthritis and rheumatism in the Gila River Indian Community .....	455
Cross-sectional and longitudinal study of "prediabetes" in the Pima Indians .....	456
Insulin resistance and the regulation of muscle glycogen synthase activity .....	457
Energy expenditures in Pima Indians: risk factors for body weight gain .....	458
WHO collaborating center for epidemiological and clinical investigations in Diabetes .....	459
Treatment of impaired glucose tolerance in Malmöhus County, Sweden .....	460
Regulation of carbohydrate and energy metabolism in human muscle .....	461
Role of insulin receptor tyrosine kinase in insulin resistance in Pima Indians .....	462
Genetics of non-insulin dependent diabetes mellitus .....	463
Regulation of skeletal muscle ribosomal protein S6 kinase by insulin .....	464
Contribution of protein tyrosine phosphatase to insulin resistance .....	465
Regulation of pyruvate kinase by insulin .....	466
Regulation of skeletal muscle casein kinase II by insulin .....	467
Relationship between insulin resistance and blood pressure .....	468

The long Q-T interval syndrome in diabetes mellitus .....	469
Autonomic nervous system activity in obesity .....	470
Epidemiology of complications of non-insulin-dependent diabetes .....	471
Kidney function in non-insulin-dependent diabetes mellitus .....	472
Insulin and hypertension in Pima Indians .....	473
Dietary survey of the Pima Indians of the Gila River Indian Community .....	474
Sodium - lithium countertransport and blood pressure .....	475
Insulin resistance in obesity and the association with lymph insulin kinetics .....	476
Effect of nicotinic acid-induced insulin resistance on $\beta$ -cell function .....	477
Regulation of gene expression by insulin .....	478

PROJECT NUMBERS

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND  
KIDNEY DISEASES

ACTIVE PROJECTS

Z01 DK 13001-17 MRB  
Z01 DK 13002-18 MRB  
Z01 DK 13004-16 MRB  
Z01 DK 13020-01 MRB  
Z01 DK 15005-15 LCDB  
Z01 DK 15100-20 LCDB  
Z01 DK 15102-30 LCDB  
Z01 DK 15200-30 LCDB  
Z01 DK 15401-18 LCDB  
Z01 DK 15404-06 LCDB  
Z01 DK 15500-30 LCDB  
Z01 DK 15503-09 LCDB  
Z01 DK 15506-07 LCDB  
Z01 DK 15508-03 LCDB  
Z01 DK 17001-24 LBM  
Z01 DK 17002-20 LBM  
Z01 DK 17003-23 LBM  
Z01 DK 17004-22 LBM  
Z01 DK 17008-07 LBM  
Z01 DK 17009-05 LBM  
Z01 DK 17024-07 LBM  
Z01 DK 18007-11 LBM  
Z01 DK 18008-24 LBM  
Z01 DK 18009-11 LBM  
Z01 DK 18010-03 LBM  
Z01 DK 18011-03 LBM  
Z01 DK 18012-06 LBM  
Z01 DK 18013-03 LBM  
Z01 DK 18014-06 LBM  
Z01 DK 18015-06 LBM  
Z01 DK 18016-03 LBM  
Z01 DK 19001-18 LC  
Z01 DK 19603-14 LC  
Z01 DK 19604-20 LC  
Z01 DK 19605-14 LC  
Z01 DK 19606-14 LC  
Z01 DK 19607-08 LC  
Z01 DK 19608-07 LC  
Z01 DK 19610-03 LC  
Z01 DK 19611-03 LC  
Z01 DK 19612-01 LC  
Z01 DK 19613-01 LC  
Z01 DK 19614-01 LC  
Z01 DK 21008-24 LCBG  
Z01 DK 21019-08 LCBG  
Z01 DK 23140-32 LBP



Z01 DK 23330-12 LBP  
Z01 DK 23580-27 LBP  
Z01 DK 23750-04 LBP  
Z01 DK 24140-24 LBP  
Z01 DK 24150-19 LBP  
Z01 DK 24260-24 LBP  
Z01 DK 24590-19 LBP  
Z01 DK 24709-09 LBP  
Z01 DK 24940-17 LBP  
Z01 DK 25008-27 LCB  
Z01 DK 25011-16 LCB  
Z01 DK 25016-17 LCB  
Z01 DK 25021-15 LCB  
Z01 DK 25025-14 LCB  
Z01 DK 25028-12 LCB  
Z01 DK 25045-07 LCB  
Z01 DK 25058-05 LCB  
Z01 DK 25059-05 LCB  
Z01 DK 25060-05 LCB  
Z01 DK 25061-05 LCB  
Z01 DK 25063-04 LCB  
Z01 DK 25064-04 LCB  
Z01 DK 25066-04 LCB  
Z01 DK 25069-02 LCB  
Z01 DK 25070-02 LCB  
Z01 DK 25071-02 LCB  
Z01 DK 25073-02 LCB  
Z01 DK 25074-02 LCB  
Z01 DK 27075-02 LCB  
Z01 DK 27076-01 LCB  
Z01 DK 27077-01 LCB  
Z01 DK 29001-18 LCP  
Z01 DK 29005-16 LCP  
Z01 DK 29006-20 LCP  
Z01 DK 29007-19 LCP  
Z01 DK 29008-19 LCP  
Z01 DK 29010-18 LCP  
Z01 DK 29011-19 LCP  
Z01 DK 29016-15 LCP  
Z01 DK 29017-11 LCP  
Z01 DK 29019-10 LCP  
Z01 DK 29020-06 LCP  
Z01 DK 29021-05 LCP  
Z01 DK 29022-03 LCP  
Z01 DK 29023-03 LCP  
Z01 DK 29025-02 LCP  
Z01 DK 29026-02 LCP  
Z01 DK 29027-02 LCP  
Z01 DK 31100-25 LBC

Z01 DK 31101-22 LBC  
Z01 DK 31102-19 LBC  
Z01 DK 31104-22 LBC  
Z01 DK 31106-03 LBC  
Z01 DK 31107-03 LBC  
Z01 DK 31108-02 LBC  
Z01 DK 31109-01 LBC  
Z01 DK 33000-24 LMB  
Z01 DK 33001-06 LMB  
Z01 DK 33006-12 LMB  
Z01 DK 34001-25 LMB  
Z01 DK 34002-26 LMB  
Z01 DK 34003-22 LMB  
Z01 DK 35000-26 LMB  
Z01 DK 35050-19 LMB  
Z01 DK 36003-06 LMB  
Z01 DK 36051-22 LMB  
Z01 DK 36101-16 LMB  
Z01 DK 36102-19 LMB  
Z01 DK 36104-09 LMB  
Z01 DK 36105-08 LMB  
Z01 DK 36106-03 LMB  
Z01 DK 36108-03 LMB  
Z01 DK 36109-03 LMB  
Z01 DK 36110-02 LMB  
Z01 DK 36111-02 LMB  
Z01 DK 36112-02 LMB  
Z01 DK 36113-01 LMB  
Z01 DK 43002-25 MDB  
Z01 DK 43003-25 MDB  
Z01 DK 43006-15 MDB  
Z01 DK 43008-09 MDB  
Z01 DK 43009-05 MDB  
Z01 DK 43200-11 MDB  
Z01 DK 43201-06 MDB  
Z01 DK 43202-07 MDB  
Z01 DK 43204-10 MDB  
Z01 DK 43205-13 MDB  
Z01 DK 43210-06 MDB  
Z01 DK 43211-06 MDB  
Z01 DK 43217-06 MDB  
Z01 DK 43221-05 MDB  
Z01 DK 43222-05 MDB  
Z01 DK 43224-04 MDB  
Z01 DK 43225-03 MDB  
Z01 DK 43227-03 MDB  
Z01 DK 43230-02 MDB  
Z01 DK 43231-02 MDB  
Z01 DK 43232-01 MDB  
Z01 DK 43233-01 MDB  
Z01 DK 43234-01 MDB  
Z01 DK 43235-01 MDB  
Z01 DK 45000-23 CEB

Z01 DK 45009-23 CEB  
Z01 DK 45014-19 CEB  
Z01 DK 45016-20 CEB  
Z01 DK 45018-15 CEB  
Z01 DK 45028-12 CEB  
Z01 DK 45033-07 CEB  
Z01 DK 45038-03 CEB  
Z01 DK 45040-02 CEB  
Z01 DK 45041-01 CEB  
Z01 DK 45042-01 CEB  
Z01 DK 47001-09 DB  
Z01 DK 47002-03 DB  
Z01 DK 47005-18 DB  
Z01 DK 47009-03 DB  
Z01 DK 47014-21 DB  
Z01 DK 47018-13 DB  
Z01 DK 47019-13 DB  
Z01 DK 47022-11 DB  
Z01 DK 47024-11 DB  
Z01 DK 47025-07 DB  
Z01 DK 47026-06 DB  
Z01 DK 47027-05 DB  
Z01 DK 47028-01 DB  
Z01 DK 51000-32 CHB  
Z01 DK 51001-32 CHB  
Z01 DK 52008-11 GBB  
Z01 DK 52011-06 GBB  
Z01 DK 52012-06 GBB  
Z01 DK 52014-03 GBB  
Z01 DK 52015-02 GBB  
Z01 DK 53001-20 DDB  
Z01 DK 53002-18 DDB  
Z01 DK 53004-18 DDB  
Z01 DK 53100-02 DDB  
Z01 DK 53101-02 DDB  
Z01 DK 53200-01 DDB  
Z01 DK 53201-01 DDB  
Z01 DK 53501-17 DDB  
Z01 DK 53503-16 DDB  
Z01 DK 53509-12 DDB  
Z01 DK 53510-11 DDB  
Z01 DK 53511-11 DDB  
Z01 DK 53515-04 DDB  
Z01 DK 53516-01 DDB  
Z01 DK 55000-18 MCNE  
Z01 DK 55002-10 MCNE  
Z01 DK 55006-17 MCNE  
Z01 DK 55007-12 MCNE  
Z01 DK 55008-12 MCNE  
Z01 DK 55010-09 MCNE  
Z01 DK 55012-08 MCNE  
Z01 DK 55013-07 MCNE  
Z01 DK 55014-07 MCNE  
Z01 DK 55015-01 MCNE  
Z01 DK 57000-25 LSB

Z01 DK 57001-13 LSB  
Z01 DK 57002-16 LSB  
Z01 DK 57501-14 LMCB  
Z01 DK 57502-17 LMCB  
Z01 DK 57503-17 LMCB  
Z01 DK 57504-03 LMCB  
Z01 DK 58000-45 LAC  
Z01 DK 58002-15 LAC  
Z01 DK 58003-17 LAC  
Z01 DK 58004-23 LAC  
Z01 DK 58005-17 LAC  
Z01 DK 58007-06 LAC  
Z01 DK 58010-05 LAC  
Z01 DK 58011-14 LAC  
Z01 DK 58014-03 LAC  
Z01 DK 58017-01 LAC  
Z01 DK 58018-01 LAC  
Z01 DK 58501-04 LNS  
Z01 DK 59000-03 MPB  
Z01 DK 59001-25 MPB  
Z01 DK 59002-25 MPB  
Z01 DK 59501-04 LMC  
Z01 DK 59502-04 LMC  
Z01 DK 69000-25 PECR  
Z01 DK 69001-21 PECR  
Z01 DK 69006-20 PECR  
Z01 DK 69009-25 PECR  
Z01 DK 69015-08 PECR  
Z01 DK 69020-07 PECR  
Z01 DK 69021-10 PECR  
Z01 DK 69024-04 PECR  
Z01 DK 69025-04 PECR  
Z01 DK 69026-04 PECR  
Z01 DK 69027-03 PECR  
Z01 DK 69028-02 PECR  
Z01 DK 69029-02 PECR  
Z01 DK 69030-02 PECR  
Z01 DK 69031-02 PECR  
Z01 DK 69032-02 PECR  
Z01 DK 69033-02 PECR  
Z01 DK 69034-02 PECR  
Z01 DK 69035-02 PECR  
Z01 DK 69036-01 PECR  
Z01 DK 69037-01 PECR  
Z01 DK 69038-01 PECR  
Z01 DK 69039-01 PECR  
Z01 DK 69040-01 PECR  
Z01 DK 69041-01 PECR  
Z01 DK 69042-01 PECR  
Z01 DK 69043-01 PECR

INACTIVE PROJECTS

Z01 DK 43228-03 MDB  
Z01 DK 45034-07 CEB  
Z01 DK 47007-15 DB

# TERMINATED PROJECTS

Z01 DK 13014-09 MRB  
Z01 DK 13017-07 MRB  
Z01 DK 15004-15 LCDB  
Z01 DK 15400-16 LCDB  
Z01 DK 25038-10 LCB  
Z01 DK 25056-06 LCB  
Z01 DK 25057-06 LCB  
Z01 DK 25068-04 LCB  
Z01 DK 25072-02 LCB  
Z01 DK 29002-16 LCP  
Z01 DK 29009-16 LCP  
Z01 DK 31105-05 LBC  
Z01 DK 33002-04 LMB  
Z01 DK 43220-05 MDB  
Z01 DK 43226-03 MDB  
Z01 DK 43229-02 MDB  
Z01 DK 45004-19 CEB  
Z01 DK 53505-15 DDB  
Z01 DK 53508-13 DDB  
Z01 DK 53514-07 DDB  
Z01 DK 55001-14 MCNEB  
Z01 DK 58001-17 LAC  
Z01 DK 58006-07 LAC  
Z01 DK 58012-04 LAC  
Z01 DK 58013-04 LAC  
Z01 DK 58015-03 LAC  
Z01 DK 58016-02 LAC  
Z01 DK 69003-17 PECR



Annual Report of the  
Mathematical Research Branch

National Institute of Diabetes and Digestive and Kidney Diseases

Current research projects of the Mathematical Research Branch reflect a broad range of interests in the development and application of theoretical models and of quantitative methodologies for understanding biological systems.

This research involves several different collaborations within the Branch and with other research groups, both at the NIH and elsewhere. This report describes recent work on cellular and network neurobiology, electrical activity of secretory cells, microcirculation and facilitated transport, renal and auditory physiology, and selected other topics.

During the past year, international collaborative projects have involved foreign investigators at Hebrew University, Jerusalem (Department of Neurobiology) and at the University Nacional Autonoma de Mexico, Mexico City (Department of Mathematics); MRB scientists participated in securing NSF Binational grants for these projects. Invited presentations at distinguished symposia were given by J Rinzel (Dynamical Systems, Orlando and Mathematical Approaches to Cardiac Arrhythmias, New York), by W Rall (Segmental Motor System, Tucson), and by GB Ermentrout (Workshop on Neuronal Oscillators, Jacksonville). Several MRB scientists were invited lecturers at the intensive course on Computational Neuronal Modeling (Marine Biological Laboratory, Woods Hole), at the workshop on Analysis and Modeling of Neural Systems (Univ California, Berkeley), and at the Workshop on Nonlinear Mechanisms of Insulin Secretion (Univ California, Davis). Invited overviews for specialized volumes were written by J Rinzel (Classics in Theoretical Biology, Bull Math Biol) and by W Rall (The Neurosciences: Paths of Discovery). J Rinzel taught a one-semester graduate-level course on Nonlinear Dynamics in Biology (Dept of Math, Univ Maryland, College Park). MRB scientists serve on the Editorial Boards of seven scientific journals.

Cellular Neurobiology

Long-term potentiation (LTP). The induction of long-term potentiation is thought to depend on  $Ca^{2+}$  influx through NMDA receptor channels. We continued to revise our model of  $Ca^{2+}$  influx through NMDA receptor channels on spines of a hippocampal dentate granule cell. In our previous model all of the activated synapses were on spines located within 100  $\mu m$  of each other on a single dendrite. We have generalized the model so that synapses on any dendrite could be activated. Others have found experimentally that spatial convergence of weak and strong inputs is necessary to produce associative potentiation of a weak input to the dentate gyrus. Our model fails to reproduce this experimental result; the model predicts a cooperative interaction between strong and weak inputs that are spatially separated on the outer and inner thirds of the dendritic tree. We have modified the model to include inhibition, but to date we have not been able to resolve the discrepancy. (WR Holmes and WB Levy: Univ. VA)

Dendritic spines. Previous theoretical investigations have focused on the electrical resistance of the spine neck. However, the neck also provides a large diffusional resistance. The importance of this diffusional resistance was studied with models. For certain synaptic calcium currents, peak spine head calcium concentration was ten times larger in long, thin spines than in short, stubby or mushroom-shaped spines. Although the diffusional resistance of the spine neck was important for producing these differences in spine head calcium concentration, the amplitude and duration of the calcium current relative to the number of calcium binding sites determined whether calcium would become highly concentrated. Given the importance of calcium for long-term potentiation, the ability of spines to concentrate calcium may play a key role in processes leading to learning and memory storage. (WR Holmes)

Estimating the electrotonic structure of neurons. Work has continued on finding efficient ways to estimate the electrotonic structure of neurons whose dendritic trees cannot be approximated as equivalent cylinders. In particular we have explored how a "reduced" model might be used to get preliminary estimates of the electrotonic parameters which can then be used to solve the "inverse" model using all of the known morphological information. A number of explicit examples have been developed to illustrate the approach. (WR Holmes and W Rall).

Thalamic neurons. Our Hodgkin-Huxley-like model for the low threshold spike (LTS) in thalamic neurons has been reformulated and extended. Recent whole-cell voltage clamp data are used to fit the model. We confirm suspicions that the LTS is due primarily to deinactivation of the transient low-threshold (T-type) calcium current. By including a three-state inactivation gate in our latest model, we also account for the slow recovery (time scale, 100 msec) of the LTS and T-channel inactivation. This slow process and the LTS are thought to play crucial roles in the synchronized 10 Hz oscillations of the thalamo-cortical system during drowsiness or quiet sleep. To study synchronization we are developing models for inhibitory synaptic interactions among cells which possess T-type channels. (XJ Wang, J Rinzel and M Rogawski: NINDS)

Learning and memory in Aplysia. Progress has been made in understanding the cellular and molecular mechanisms for two non-associative learning processes in Aplysia californica, habituation and sensitization of the gill withdrawal reflex. The two long-lasting modifications involve biochemical transformation of the siphon sensory neuron to gill motor neuron synapse, modulation of a particular potassium current through phosphorylation of the channel by protein kinase, and activation of this protein kinase by cyclic AMP (whose synthesis is turned on by tail stimulation). Based on the collective experimental evidence we have developed a mathematical model for the currents of the siphon sensory neuron and for the memory traces in the synapse. Our simulations have shown that slow voltage-dependent inactivation of the calcium current underlies habituation of the response. On the other hand, during sensitization, the action potential broadens (due to phosphorylation of the potassium channel), and this permits a larger increase in the intracellular calcium level during spiking and greater neurotransmitter release. A model for the regulation of the phosphorylation dephosphorylation cycle has also been considered. It predicts that: (a) the siphon stimuli, when paired with tail excitations, enhance sensitization; (b) the sensitization dependent loss



of the kinase's regulatory subunit provides a mechanism for long term (weeks) retention of the memory at the synaptic level. (JL Martiel)

Slow-fast analysis of bursting and synaptic coupling. We continue to develop and apply the averaging method to differential equation models of multiple time scale phenomena. In this technique one averages the effects of rapid action potentials on the slow processes, and then a reduced model for only the "averaged" slow processes is studied. One application is to endogenous neuronal bursting oscillations (with S Baer: Arizona State Univ. and H Carillo-Calvet: UNAM, Mexico City). We find that the averaged equations, for our minimal model of parabolic bursting, approximate well the slow variable time courses of the full model. In different parameter regimes we identify resting or bursting or continuous spiking behaviors, as found in neuronal bursters. Some counterintuitive results for the minimal model are leading to new insights on more complex, biophysically realistic, models. A second application is to neuronal interactions mediated by slow synapses, as in recent tissue culture studies of Aplysia neurons. With P Frankel (Brown Univ) a set of models has been formulated which allows comparisons between complete descriptions, with explicit action potentials, and simpler averaged equations for synaptic inputs as the slow variables. Several dynamic behaviors of the experimental network model are reproduced. (J Rinzel)

### Network Neurobiology

Flicker enhancement in vision. With the use of flickering test stimuli intracellular recordings have been made from cat retinal horizontal cells. Dim backgrounds increase response amplitudes to small but not large stimuli. Such enhancements, measured for different widths of slit and square stimuli, are compared with theoretical results for two different spatial models. In the "dark test-region" model rods within the test region are assumed unresponsive to background stimuli because of prior saturation by the test stimulus. Background-evoked rod signals gradually decrease from regions outside the test stimulus through a syncytial network into the recording site, where they act on the cone-to-horizontal-cell synapse, increasing its gain. In the "changing length-constant" model rod signals reduce the length constant of a syncytial network by uncoupling the cells within it. This causes an increased response to small but not large test stimuli. Both models are solved analytically, using the conductive-sheet approximation to the syncytial network, to predict response enhancement as a function of stimulus size and shape. The first model exhibits exponential decay of flicker enhancement as a function of slit width but a steeper than exponential decay with the width of squares, as in experiments. The second model makes similar predictions, however the decline of flicker enhancement with size of square test stimuli is somewhat shallower than for either the dark test-region model or for the experiments. (SM Baer: MRB; R Nelson, R Pflug: NINDS)

Inhibition induced symmetry creation in an oscillatory neural net. A network of  $N$  excitatory cells is coupled to a single inhibitory cell. If the inhibition is fast, the network acts as a winner-take-all net and a single cell is excited at the expense of the others. As the inhibition is slowed down, the WTA solution loses stability to an oscillating solution. There are  $2N$  such solutions that merge via an  $N$ -fold saddle-node. This leads to complex oscillatory behavior that resembles bursting. As the inhibition is slowed down even further, this behavior collapses to a symmetric synchronous periodic

Dendritic spines. Previous theoretical investigations have focused on the electrical resistance of the spine neck. However, the neck also provides a large diffusional resistance. The importance of this diffusional resistance was studied with models. For certain synaptic calcium currents, peak spine head calcium concentration was ten times larger in long, thin spines than in short, stubby or mushroom-shaped spines. Although the diffusional resistance of the spine neck was important for producing these differences in spine head calcium concentration, the amplitude and duration of the calcium current relative to the number of calcium binding sites determined whether calcium would become highly concentrated. Given the importance of calcium for long-term potentiation, the ability of spines to concentrate calcium may play a key role in processes leading to learning and memory storage. (WR Holmes)

Estimating the electrotonic structure of neurons. Work has continued on finding efficient ways to estimate the electrotonic structure of neurons whose dendritic trees cannot be approximated as equivalent cylinders. In particular we have explored how a "reduced" model might be used to get preliminary estimates of the electrotonic parameters which can then be used to solve the "inverse" model using all of the known morphological information. A number of explicit examples have been developed to illustrate the approach. (WR Holmes and W Rall).

Thalamic neurons. Our Hodgkin-Huxley-like model for the low threshold spike (LTS) in thalamic neurons has been reformulated and extended. Recent whole-cell voltage clamp data are used to fit the model. We confirm suspicions that the LTS is due primarily to deinactivation of the transient low-threshold (T-type) calcium current. By including a three-state inactivation gate in our latest model, we also account for the slow recovery (time scale, 100 msec) of the LTS and T-channel inactivation. This slow process and the LTS are thought to play crucial roles in the synchronized 10 Hz oscillations of the thalamo-cortical system during drowsiness or quiet sleep. To study synchronization we are developing models for inhibitory synaptic interactions among cells which possess T-type channels. (XJ Wang, J Rinzel and M Rogawski: NINDS)

Learning and memory in Aplysia. Progress has been made in understanding the cellular and molecular mechanisms for two non-associative learning processes in Aplysia californica, habituation and sensitization of the gill withdrawal reflex. The two long-lasting modifications involve biochemical transformation of the siphon sensory neuron to gill motor neuron synapse, modulation of a particular potassium current through phosphorylation of the channel by protein kinase, and activation of this protein kinase by cyclic AMP (whose synthesis is turned on by tail stimulation). Based on the collective experimental evidence we have developed a mathematical model for the currents of the siphon sensory neuron and for the memory traces in the synapse. Our simulations have shown that slow voltage-dependent inactivation of the calcium current underlies habituation of the response. On the other hand, during sensitization, the action potential broadens (due to phosphorylation of the potassium channel), and this permits a larger increase in the intracellular calcium level during spiking and greater neurotransmitter release. A model for the regulation of the phosphorylation dephosphorylation cycle has also been considered. It predicts that: (a) the siphon stimuli, when paired with tail excitations, enhance sensitization; (b) the sensitization dependent loss

Synchronization by ionic coupling. While limited electrical coupling of beta cells via gap junctions has been demonstrated, its extent is unknown. However, the beta cells in an islet also share an extracellular compartment in which potassium ion concentration (K) oscillates synchronously with electrical bursting. Since the ratio of external to internal K strongly affects membrane potential, we have developed a theoretical model to investigate the possible role of extracellular K diffusion in synchronization of beta cell electrical oscillations. Using reasonable parameter values, we find that cells synchronize very quickly (within one burst), without any gap junctional coupling. The shape and amplitude of computed K oscillations resemble those seen in experiments. The model cells synchronize with exterior cells leading. In experiments, either the interior or exterior cells led. Some extensions of the model (e.g., allowing cells to have non-identical membrane properties) can account for such variability. Mathematical analysis of the mechanism of synchronization in the model is underway. (CS Stokes and J Rinzel)

Calcium inactivation of calcium channels. Others have proposed that the time course of inactivation of whole-cell Ca current results from accumulation of Ca ions in a macroscopic sub-membrane "shell". Motivated by calculations showing that channel density in the pancreatic beta-cell is too low to generate a shell, we have proposed instead that Ca ions in the local domain under an open channel block only that channel. Our model produces U-shaped inactivation curves, even though whole-cell current plays no role, casting doubt on previous inferences supporting the shell model. Our model is also consistent with hitherto puzzling observations by Lux and Brown on single Ca channels which cannot be explained by the shell model. Since they examined Ca channels in a variety of tissues, our model may be generally applicable. (A Sherman, J Keizer, U-C Davis, and J Rinzel)

### Microcirculation

Synchronization of vascular contractions. A model that reproduces the periodic changes in the diameter of vascular vessels has been developed. The formulation incorporates potassium and calcium transport through voltage-gated channels in the smooth muscle cell membrane, muscle contraction through the calcium-calmodulin phosphorylation mechanism, and the resulting stress-strain changes of the vascular wall. By incorporating the experimentally found dependence of channel conductances on stress, we were able to reproduce the experimental results between changes in intraluminal pressure and oscillatory activity of small cerebral arteries. A flow network for the microcirculation was formulated; for some parameter values, synchronization between two terminal arterioles was obtained. This may be relevant to the synchronization found in the microcirculatory bed of sickle cell anemia patients (J M Gonzalez-Fernandez and GB Ermentrout).

### Renal Physiology

Tubule pH balance. Previously, we described a canonical tubule model for solute, flow, and charge conservation including individual reactive species and chemical buffers. We simulated perfusion experiments of isolated rabbit cortical collecting ducts, and we described a tubule embedded in an interstitium. Solution profiles including non-reactive ions, neutral species and electrical potential, were obtained as functions of an interstitial profile that is described analytically. Transport properties of the thick

orbit. The results are shown to mimic some of the complex patterns observed in models of hippocampal epilepsy. (GB Ermentrout)

Mass action rules for creating cortical maps. By using the simple kinetic rules:  $C + n' \rightarrow C' + n$  where  $C, C'$  are connections and  $n, n'$  are free neurites, and the rate constant  $K$  depends on  $C, C'$ , we can show that a variety of cortical maps can spontaneously appear. (i) If one assumes there is a "stickiness" gradient for neurites arising from the retina and a similar one on the tectum, then one can derive a simple integral equation from this kinetic scheme. Using similarity transforms, this reduces to a linear eigenvalue problem which can be solved for some cases. Under general circumstances, Simon Eveson (University of Sussex) proved that positive solutions to this integral equation exist. These imply the existence of an "identity" map from the retina to the tectum which is the desired outcome. (ii) If one assumes a Hebbian type of cooperativity, then ocular dominance stripes and orientation columns can spontaneously form. This is proven by using bifurcation methods. (GB Ermentrout: MRB, GF Oster: UC-Berkeley, and SE Fraser: UC-Irvine)

### Electrical Activity of Insulin-secreting Cells.

Coupling and synchronization by gap junctions. Our previous model considered the limiting case in which cell clusters are so tightly coupled that they are perfectly synchronized and they can be treated as a single cell (or "supercell") with an enlarged membrane. We now have a true "multicell" model in which identifiable, individual cells (as many as 1000) are coupled, with junctional conductance as a parameter. Using the supercomputer resources of the NCI ASCL and the DCRT we have been able to explore the effect of junctional conductance on macroscopic parameters of the bursting activity. We find that moderate conductance can synchronize cell clusters and that the burst period and amplitude of intracellular calcium oscillations are optimized by coupling conductance in a range, 150-250 pS, which corresponds well with recent experimental data on gap junctions. Interestingly, the maximal period and calcium levels exceed those for the supercell. While experimental techniques have so far allowed simultaneous measurement of membrane potential in only two cells, the model allows us to visualize an entire two-dimensional array of cells as it evolves in time. We have learned that synchronization in the model is achieved by waves of depolarization and repolarization. We hope this will motivate experiments on wave propagation in monolayer cultures. (A Sherman and J Rinzel)

Dependence of burst duration on coupling. Work on the above multicell model led to an apparent paradox: electrotonic theory predicts that burst duration should increase monotonically with junctional conductance, whereas the numerical simulations showed that burst duration first increases then decreases. We have resolved this question by considering a simplified model in which just two cells are coupled. Coupling causes a bifurcation to a new solution of the equations with longer burst period, but as coupling is increased the old, shorter period solution is restored. The balance between these effects and the electrotonic effects leads to the observed maximum. The enhancement of burst period with appropriate coupling is a collective property that has broader implications for ensembles of cellular oscillators. (A Sherman and J Rinzel)

linear superposition allows the conceptual decomposition of the branches from the branch points. This allows straight branches to be solved optimally and the dense linear system for the branch points to be solved with another, more appropriate method. This algorithm is easily implemented on many emerging advanced computer architectures. Moreover, our method (unlike its competitors) can handle geometries that contain either physical or electrical loops. (2) Solution of boundary value problems (BVPs) for elliptic PDEs. The previous report described our elegant massively parallel implementation of Wiener integral (probabilistic) representations for elliptic BVPs. The method's main drawback is the lack of multiprocessor load balancing. Our latest implementation rectifies this, and moreover it also uses the trade off of increased memory to achieve an algorithm that requires no interprocessor communication. (3) Solution of linear algebraic equations. A local pivoting variation of Gaussian elimination is being implemented on a logic programmable systolic array multiprocessor to assess the numerical computational capabilities of such a system. This is relevant to anticipated implementation of the new INMOS transputer and Intel iWarp processor chips for parallel computation. (MV Mascagni)

An adaptive model for firefly synchrony. *Pteroptyx malacciae*, a tropical Asian firefly can synchronize to light pulses with zero phase-shift over a range of frequencies. These insects congregate in trees and flash with almost perfect synchrony. In an earlier paper with John Rinzel, we showed that this could be understood via a simple "phase" model. Unfortunately, such models give a null phase-lag only if all oscillators have the same intrinsic frequency. The present work shows that by including a slow adaptive frequency term in the equations, one can prove that synchrony to a periodic stimulus with null phase lag can be obtained. For "all-to-all" coupling, similar results obtain in large congregations. This work was motivated by conversations with John Buck and Frank Hanson. (GB Ermentrout)

Entropy and information flow in large-scale dynamical systems. We introduced the notion of epsilon-entropy to study deterministic dynamical systems with many degrees of freedom. With this we relate the behavior of such systems to stochastic or statistical ones, and we extend the application of ergodic theory for low-order dynamical systems and chaos to such processes. This allowed us to classify many different types of large-scale dynamic and stochastic processes. We also applied our ideas to recent data (A Libchaber, U Chicago) on fully developed fluid turbulence. In addition, we have considered a formal neural network from this point of view. It was shown that the spontaneous oscillatory activity in this net is intermediate between low-dimensional chaos and noise. Kolmogorov and Shannon originally proposed epsilon-entropy in stochastic information theory. We wish to explore this notion in the context of "computation" by single neurons and neural networks. (XJ Wang and P Gaspard: Free Univ of Brussels, Belgium)

Nuclear magnetic resonance. Spectral localization by imaging (SLIM) (introduced by Hu, et al) is a method for acquiring simultaneous NMR spectra from several arbitrarily shaped and placed voxels. An image is acquired for spatial coordinates, and then a judicious set of phase-encoded full-induction decays (FIDs) are collected from which the voxel spectra are reconstructed using a least-squares fit. SLIM does not suffer from "bleeding" artifact between voxels and can be performed with a minimal number of phase-encode (PE) steps. Since the choice of PEed FIDs is critical for obtaining good

ascending limb of Henle (TAL) were studied. We have now shown that even if  $\text{NH}_3$  permeability is relatively large, an alkaline pH disequilibrium can block  $\text{NH}_3$  backflux in TAL. However, with  $\text{H}^+$  secretion as in rat TAL, this mechanism will not serve to sustain net total ammonia reabsorption. This suggests a low  $\text{NH}_3$  permeability in rat TAL, since reabsorption is necessary for  $\text{NH}_4$  concentration via a countercurrent mechanism. (R Mejia and M Knepper: NHLBI)

Acid/base balance in the kidney. We are presently developing a multinephron model of acid/base balance for the whole kidney. To solve the large nonlinear system of differential-algebraic equations, we have developed a 4th-order-accurate, variable-step, space discretization scheme with root-finding. Use of domain decomposition reduces the problem to solution of 10 discretized differential equations and 8 algebraic equations at each space (and time) position in each nephron segment, leaving approximately 2000 algebraic equations for the interstitium and boundaries. Parameter continuation permits development of a solution for a set of thermodynamic parameters (or boundary values) from a previous solution. (R Mejia and M Knepper: NHLBI)

### Auditory Physiology

Representation of the acoustic spectrum in the auditory cortex. This project combines neurophysiological mappings of the ferret primary auditory cortex and mathematical models of the data. Our experiments have attempted to measure the changes in the character of the receptive fields along the isofrequency planes and to identify the functional significance of these changes. We have collected data from over 20 animals and used them to generate models of the receptive fields in the cortex. The distribution of activity across the surface of the cortex is then computed for many complex spectra. Our results so far support the discovery of a columnar organization that parallels the orientation columns of the visual cortex. Specifically, the isofrequency planes seem to distribute the responses according to the gradient of the spectral profile at each frequency (SA Shamma).

Adaptive pattern recognition with neural networks. This project is now almost complete. The work involved the formulation of a learning algorithm that is unsupervised and computationally efficient. The algorithm is based on a gradient decent formulation, and has been demonstrated in a variety of useful applications ranging from the segmentation of speech, to the extraction of pitch, and the classification of phonemes. (SA Shamma)

Formulation of concise, mathematically tractable descriptions of the essential minimal signal processing steps needed to mimic cochlear function. These are being achieved through the application of wavelet transform methods and multiscale processing theory to the analysis of the cochlear stages and early auditory networks. The primary goal here is to provide a deeper understanding of the way cochlear models enhance both the representation of the acoustic features and their robustness in noisy environments. (SA Shamma)

### Selected Other Topics

Application of high performance computing to numerical problems. (1) Solution of coupled Hodgkin-Huxley-like partial differential equation (PDE) models that include neuronal branching geometries. Our new algorithm based on

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,001-17 MRB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical formulations and analysis relevant to experimental neurophysiology.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. Rall Senior Research Physicist MRB, NIDDK

Other: W. R. Holmes Staff Fellow MRB, NIDDK

## COOPERATING UNITS (if any)

Dept. of Neuroscience, Hebrew univ. of Jerusalem

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.8

## PROFESSIONAL:

2.5

## OTHER:

.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

RESEARCH AREA. Basic neuroscience involving structure/function relations for neuronal dendritic branching, dendritic spines, and synapses (also neuron populations, with cortical symmetry), and for such functions as synaptic transmission, amplification and dendro-dendritic interactions in the context of spatio-temporal input patterns, logical processing of input, and neural plasticity, as in conditioning and learning.

RATIONALE. Combine experimental data from neuroanatomy and from electrophysiology with biophysical models of nerve membrane (passive, synaptic and excitable) into a comprehensive theory which can lead to new insights and to testable theoretical predictions (leading to the design of better experiments). To do this we must create, explore and test mathematical and computational models with different degrees of complexity.

METHODOLOGY. Our methods include both analytical solutions and computational solutions of boundary value problems (for partial differential equations) in the tradition of classical physics. They include also the formulation and solution of problems in terms of systems of ordinary differential equations; when this is done explicitly for a compartmental model of a neuron, it is possible to accommodate a remarkable variety of dendritic branching patterns and non-uniform distributions of membrane properties and of synaptic inputs.

RESULTS. Summarized in Chapt. 3 of "The Handbook of Physiology: The Nervous System, Vol. 1" American Physiological Society, 1977 (Kandel, Brookhart & Mountcastle, eds.) and in Ch. 22, 24 in "Cellular Mechanisms of Conditioning and Behavioral Plasticity" (eds. Woody, CD, Alkon, DL & McGaugh, JL) Plenum Press, 1988.

localization and signal-to-noise ratio (S/N), we have sought to develop a numerical procedure to optimize the PE gradient steps. We have evaluated a criterion based on the product of individual criteria for localization and S/N in a multidimensional optimization algorithm. We have demonstrated the utility of this optimized SLIM in a study of the rabbit kidney where highly localized  $^1\text{H}$  NMR-detectable metabolites are present. (von Kienlin, R Mejia and RS Balaban: NHLBI)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 13,004-16 MRB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical description of cellular neuroelectric signal transmission.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Rinzel	Chief, MRB	MRB, NIDDK
Other:	A. S. Sherman	Senior Staff Fellow	MRB, NIDDK
	C. Stokes	NRC Fellow	MRB, NIDDK
	X. J. Wang	Fogarty Fellow	MRB, NIDDK

COOPERATING UNITS (if any)

Lab/Cell Biol & Genetics, NIDDK;	Dept/Mathematics, U/Pittsburgh
Dept/Chem, U/California, Davis;	Dept/Mathematics, UNAM, Mexico City, Mex.
Dept/Mathematics, Arizona St. Univ.	Med. Neurology Br, NINCDS

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.5

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of electrical activity of neurons and other cell types. Among the topics of current interest are: (i) integration of synaptic input delivered to the soma and dendritic branches of a neuron; (ii) propagation of action potentials along axons; (iii) stimulus-response and threshold properties for repetitive-firing of action potentials; (iv) complex bursting patterns of membrane potential oscillations which arise through endogenous membrane properties and/or intercellular coupling.

Because qualitatively related mathematical or biophysical problems may arise in other context, e.g. chemical and biochemical oscillations, or e.g. excitation-secretion coupling, this project may consider models from such applications.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods drawn from both classical and modern applied mathematics. These methods may include finite difference or finite element numerical integration, bifurcation theory, perturbation techniques, and nonlinear dynamical systems theory. One goal of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet physiologically reasonable, equations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,002-18 MRB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical description of substrate transport in capillary-tissue structures.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. M. Gonzalez-Fernandez

Research Mathematician

MRB, NIDDK

## COOPERATING UNITS (if any)

Dept. of Mathematics  
Univ. of Pittsburgh

Lab. of Chemical Biology, NIDDK

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.53

## PROFESSIONAL:

1.5

## OTHER:

.03

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The goal of this work is to develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models of the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,017-07 MRB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sound processing in the auditory system.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:

S. A. Shamma

Guest Worker

MRB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13.014-09 MRB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Probabilistic Analyses of Nucleic Acid Sequences.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. J. Lipman

Research Scientist

MRB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The thirty-five scientists and support personnel of the Laboratory of Cellular and Developmental Biology are a highly diverse group, much like a small university department. Their investigations span a broad spectrum of scientific interests. At one end of this spectrum are atomic and molecular resolution structural studies of proteins and protein-DNA complexes; at the other are studies of mammalian development and genetic defects. Between these are groups interested in genetics and gene regulation in yeast, *Dictyostelium discoideum*, sea urchins and human tissue culture cells; ultrastructure; biotechnology; hormonal regulation mechanisms and signal transduction. Most of the groups share common technological approaches in their work; this, together with an environment which fosters communication, leads to highly productive interactions among all groups within the laboratory, in spite of the seeming diversity of research areas under investigation.

The past year has seen significant progress in all the areas studied in LCDB. In addition to research papers, members of the laboratory have published critical invited reviews and presented data at universities, other NIH laboratories, scientific companies and national and international meetings.

Dr. Michael Lin, a member of LCDB from the time he came to work with Dr. Martin Rodbell, moved to the extramural program of NHLBI this year. Dr. Lin is now applying his extensive experience and knowledge in hormonal regulation, growth factors, and oncogene products to research in hypertension and cardiovascular diseases. Dr. Herbert Windmueller, another long time laboratory member until his retirement for health reasons two years ago, succumbed to amyotrophic lateral sclerosis in 1989. Herb had been a widely recognized expert in the role of the small intestine in glutamine and apolipoprotein metabolism. His contributions are missed by the scientific community and his gentle, thoughtful interactions leave a void for those of us who knew him well before his medical problems limited his research.

The following summary of research in LCDB for 1989-1990 is organized as we have done previously. Rather than summarize experimental work done by individual Sections or working groups, I choose to review progress in a thematic sense, by the types of research done in the laboratory. This approach emphasizes the continuity of the spectrum of research done in LCDB and will hopefully lead the reader to an appreciation of the interactions which make the laboratory more than the sum of its component parts.

### Structural Studies

The use of barnase, an extracellular ribonuclease of *B. amyloliquefaciens*, and barstar, its cognate intracellular inhibitor, as a system for investigation of protein folding mechanisms and protein-protein interactions was markedly facilitated several years ago by cloning and overexpression of the barnase gene, followed in a year by similar achievements for the barstar protein. Efforts are now underway to characterize the structure of barstar and the 1:1 complex of the two proteins. A collaborative effort has been established with several European laboratories to advance study of the barnase:barstar system. The barnase crystal structure has been refined with 2.0 Å x-ray data. The structure of barstar in solution is well underway using 2-D NMR techniques in the laboratory of Dr. Jean Garnier in France, using the recombinant protein produced in our laboratory.

Recombinant barnase is identical to the native enzyme in sequence, enzymatic specificity, thermal unfolding and crystal morphology. Both native and recombinant barstar proteins have variable degrees of oxidation of the two cysteinyl residues, although any of the forms of barstar are equally effective in the inhibition of barnase activity. We reported previously that replacement of the cysteines singly or both by serines was without effect on inhibitory activity but led to a less stable protein which was produced in low yield. We recently found that replacement of the cysteinyl

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,020-01 MRB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electrical and chemical oscillations in coupled cell systems.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Sherman

Senior Staff Fellow

MRB, NIDDK

Other: J. Rinzel

Chief, MRB

MRB, NIDDK

## COOPERATING UNITS (if any)

Lab/Cell Biol &amp; Genetics, NIDDK

Dept/Chem, U/California, Davis

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.48

## PROFESSIONAL:

1.45

## OTHER:

.03

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We use mathematical models to study the mechanisms of oscillatory electrical activity arising from ion channels in cell membranes and modulated by intracellular chemical processes. We are interested in both the behavior of single cells and the ways in which cells communicate and modify each other's behavior.

Our main application has been to the biophysical basis of insulin secretion in pancreatic beta-cells. We have examined bursting oscillations in membrane potential and the role of electrical coupling between cells in the islet of Langerhans. Long term goals are to understand how the membrane dynamics interact with intracellular events to regulate secretion and to generalize to other secretory cells and neurons.

Our primary tool is the numerical solution of ordinary differential equations. We use analytical, geometrical, graphical, and numerical techniques from the mathematical theory of dynamical systems to help construct and interpret the models. Perturbation techniques are used to get analytical results in special cases.

We work with both detailed biophysical models and simplified models which are more amenable to analysis. By selecting and combining features we can distill the essence of the phenomena, derive general principles, and facilitate communication with workers in related fields.

cell types. The genomic mapping data using micrococcal nuclease are essentially identical to those for the multicopy, chimeric minichromosome. Precisely positioned nucleosomes about the operator and extend for some distance distally towards the STE6 gene in  $\alpha$ -cells. This is an important result, since the sequences which are present in the genome and in the minichromosome are totally different with the exception of about 40 bp on either side of the operator and the operator itself. Although being very dissimilar in DNA sequence, the chromatin structure of the BAR1 locus (BAR1 is another  $\alpha$ -specific gene which is repressed by  $\alpha 2$ ) is very similar to that of the STE6 gene, making the phenomenon of chromatin organization by  $\alpha 2$  likely to extend to all five genes which are repressed by this protein. How the positioning signal propagates its effect beyond the two immediately adjacent nucleosomes remains a puzzle, the answer to which is likely to be of major importance in understanding the structural organization of chromatin and its relation to function.

We have continued to distribute the model substrates for higher order chromatin structure that contain tandemly repeated nucleosome positioning DNA sequences, described several years ago, to other laboratories in the United States and overseas. These molecules are currently in use in over a dozen other sites. Dr. Timothy Richmond at the ETH in Zurich, Switzerland, continues efforts at determination of a high resolution X-ray crystal structure of the core particle of the nucleosome, using a unique DNA fragment based on the 5S rRNA positioning signal and constructed in this laboratory in a collaborative effort.

### Chromatin Organization, Transcription and Regulatory Factors

We have previously suggested that organization of the structure of chromatin might be the mechanism whereby  $\alpha 2$  serves to repress transcription of  $\alpha$ -specific genes in yeast. Location of a nucleosome adjacent to the  $\alpha 2$  binding site would place the TATA box for the downstream gene in the middle of the core particle, precisely where we previously showed that the ARS core sequence, a *cis*-acting DNA element necessary for replication, functioned only poorly. Given this hypothesis, it is of interest to ask how might the  $\alpha 2$  repressor and the MCM1 protein serve to organize chromatin structure? Data reviewed above suggested that some direct interaction of this repressive complex with nucleosome constituents might serve in this role. We have examined the structure of minichromosomes containing the  $\alpha 2$  operator in  $\alpha$ -cells where certain deletions of the amino terminal regions of histones H2B and H4 are the only expressed versions of these highly conserved proteins (the deletions have been described and were generously supplied by Dr. Michael Grunstein's laboratory). Deletion of the basic amino terminal region of H2B leads to a general disorganization of chromatin structure; the basic pattern of positioned nucleosomes around the  $\alpha 2$  operator is present, although positioning is less precise than when the wild type gene is expressed. Deletions of amino acids 4-14 of H4 do not alter the chromatin structure when compared to cells containing the wild type protein. In contrast, deletions of residues 4-19 or 4-23 alter the topological properties of minichromosomes in a fashion consistent with the loss of a nucleosome and have a more random location of micrococcal nuclease cutting sites in the regions flanking the  $\alpha 2$  operator, where nucleosomes are precisely located in  $\alpha$ -cells containing normal H4 genes. These findings are similar to the structural phenotype of the minichromosome in  $\alpha$ -cells. The data strongly suggest that interactions of the  $\alpha 2$  repressor with a conserved region of H4 histone are critical in establishment of the organized chromatin structure. In this vein, it of interest that a functional phenotype, derepression of expression of the silent mating type loci, is also associated with the 4-19 and 4-23 deletions, and requires the presence of the SIR3 gene product. It will be of interest to examine the expression of  $\alpha$ -specific genes in the H4 deletions strains having a structural phenotype and the structure of SIR3 repressed genes in the same strains which are known to have a functional phenotype. The results at hand at the moment suggest a common mechanism, organization of a repressive domain of chromatin structure, for repression of transcription of at least two yeast gene families. In the case of the  $\alpha 2$  genes, the location of a nucleosome would place the TATA box in the central region of a core particle, exactly at the site where our studies of the ARS core demonstrate blockage of access of *cis*-elements to protein factors. To test this as a mechanism of repression, we have constructed fusions of the STE6 promoter with the *E. coli* galactosidase gene; mutations of this construction will allow a direct test of our hypothesis that some eukaryotic repressors function by organizing a repressive chromatin environment.

In addition to repression of  $\alpha$ -specific genes in  $\alpha$ -cells,  $\alpha 2$  and another mating type locus encoded,

residues by alanine leads to a recombinant protein which is identical to native barstar in yield and activity; this derivative should markedly facilitate structural studies.

We have long been interested in the interactions between barnase and barstar. We are using site directed mutagenesis to address these interactions. About fifty mutants for each protein have been produced, including a number of genes with two or three mutations. Knowing the structure of barnase, most mutations are charge reversals or major changes in side chain bulk for surface residues of the protein. Most are active and inhibited by barstar. Two mutants, converting the active site his-102 to lysine or arginine, produced no secreted activity, grew slowly and were often overgrown by strains negative for the mutation. This suggests that these mutants produced a low activity barnase which was not inhibited by barstar and therefore was a leaky lethal mutation. In contrast, we have previously shown that inactive mutants gln-102, asp-102 or gly-102 did interact normally with barstar. The surface of barstar has been mapped in terms of charged residues possibly involved in protein-protein interactions; each glu and asp has been changed to lys and each lys and arg converted to glu. Only two mutants, asp-35 to lys and asp-39 to lys, produced no barstar activity, although they produced normal amounts of barstar antigen. This strongly suggests a role for these two acidic residues in the interaction with barnase.

Gene regulation in eukaryotic cells occurs in the context of chromatin, a complex of DNA, histones, and nonhistone proteins; this makes understanding of chromatin structure a prerequisite to the understanding of transcriptional regulation. Previous reports from this laboratory have documented the occurrence and, in several cases, the mechanism for sequence specific association of DNA with histone octamers, leading to what have been called positioned nucleosomes. Nucleosome positioning has been speculated to be a possible mechanism in determining the function of *cis*-acting elements in eukaryotic cells. Last year we reported the first experimental evidence to confirm this speculation. The replication origin (ARS1) of a yeast minichromosome is normally located adjacent to a positioned nucleosome. Mutations which moved this 11 base pair (bp) element within 40 bp of the pseudodyad of the nucleosome severely limited its function. We have extended this study by showing that insertion of the  $\alpha 2$  operator 50 bp clockwise of the positioned nucleosome moved the location of the nucleosome to about the  $\alpha 2$  binding site, thereby exposing the replication origin and rescuing the low copy number phenotype of the -60 deletion mutant. Importantly, the  $\alpha 2$ -60 minichromosome was present in low copy number in  $\alpha$ -cells, which do not contain the  $\alpha 2$  protein. These results demonstrate that positioning by  $\alpha 2$  (discussed in more detail below) is an active, not passive, process and that sequence alterations in the deletion mutants do not have a direct effect on ARS function, confirming the importance of chromatin structure in the activity of at least one *cis*-acting DNA element.

This finding, that *cis*-acting elements can have their availability to *trans*-acting factors inhibited when placed in a nucleosome central region, takes on particular interest from other studies of positioning in yeast. We previously reported that the  $\alpha 2$  gene product which binds as a homodimer in conjunction with a dimer of MCM1 to a 31 bp operator upstream of  $\alpha$ -cell specific genes to repress their transcription organizes chromatin structure. Positioned nucleosomes flank the  $\alpha 2$  binding site in  $\alpha$ -cells or  $\alpha/\alpha$  diploid cells; nucleosome locations in the region of the minichromosome studied are random or nearly so in  $\alpha$ -cells, where the  $\alpha 2$  protein is absent. These studies have been extended to nucleotide level resolution using primer extension mapping of chromatin structure. All the data confirm the low resolution results. The location of nucleosomes flanking the  $\alpha 2$  operator is precise to a base pair, both in micrococcal nuclease and DNaseI mapping experiments. Micrococcal nuclease cutting sites are 142-146 bp from the edge of the operator. In these regions, DNaseI cuts the minichromosome with a periodicity of about 10 bp; 13 sites are located within each nucleosome. This is the best confirmation thus far that features of the structure of the nucleosome deduced from over a decade of study of isolated or reconstituted core particles actually exist for the chromatin of a unique genetic locus *in vivo*.

We were concerned that this striking organization of chromatin structure by the  $\alpha 2$  operator binding proteins might be in part related to the multicopy nature of the yeast minichromosome or to the bacterial plasmid sequences which flank the 100 bp yeast DNA insert containing the operator in the plasmid. After optimizing polymerase amplification methodology, we carried out similar studies of the chromatin structure of the native STE6 promoter region as a single genomic copy in  $\alpha$ - and  $\alpha$ -



thought to be important for the assembly of the transcription complex for 5S rRNA.

The chromatin structure of a yeast heat shock protein gene (HSP80) has been detailed previously. We now have confirmed that this gene is also expressed during yeast sporulation and have reason to suspect that a different promoter may be involved in this expression vs. heat shock expression. A region about 1 kb 5' to the gene contains a profusion of exact or partial matches to sequences thought to be binding sites for known trans-acting transcriptional regulatory factors. We made gene substitutions which deleted the 1500 bp 5' to the gene as well as the structural gene itself in a supersporulating strain of yeast. The mutant strains have a slow growth phenotype, yielding small colonies on tetrad dissection and analysis, compared to mating progeny with the gene intact. A limited (<150 bp) deletion of the putative control region about 1 kb 5' to the gene leads to an identical phenotype, strongly suggesting the importance of this region in expression of this heat shock gene.

These studies, aimed at elucidating the structure of complexes containing previously identified *trans*-acting regulatory factors, provide a sound base for other investigations which have as their goal the identification of such *trans*-acting factors in more complex systems. One of these is the search for proteins involved in the tissue and developmental stage specific regulation of human globin genes. The gene of particular interest is the  $\epsilon$ -globin gene, a member of the  $\beta$ -globin cluster expressed during embryonic life. K562 cells, derived from a human leukemia, express this gene. Transient assays, using the chloramphenicol acetyltransferase gene as a reporter, showed that a 458 bp fragment (-274 to +184) is sufficient for function as the  $\epsilon$ -globin promoter in K562 cells. Binding of proteins in cell extracts to segments of this fragment was tested by footprinting and gel mobility shift assays. Eight distinct footprints were observed, at least two of which were erythroid specific (*i.e.*, not present with HeLa cell extracts). Some of these correspond to known *cis*-acting elements, the CCAAT and ATA motifs and an Sp1 site on the opposite strand from the CACCC globin consensus sequence. One footprint, present with both K562 and HeLa extracts, does not correspond to any known transcriptional regulatory factor. Two erythroid specific binding sites for EryF1 were identified in this region. Using erythroid and HeLa extracts, there was a one-to-one correspondence of the footprint results and gel mobility shift assays using synthetic oligonucleotides that spanned the regions of interest.

We reported previously a modest enhancement of expression of a reporter gene by a 305 bp fragment which resides 3' of the  $\epsilon$ -globin gene. The increase in activity was far smaller than that obtained with a chicken  $\beta$ -globin enhancer, necessitating cotransfection with a  $\beta$ -galactosidase gene as a control for efficiency; results of this set of experiments are pending. Nonetheless, footprinting shows six regions of this sequence which interact with proteins in K562 or HeLa cell extracts. Two consensus sites for NF-E1 are present in this DNA segment; gel mobility shift assays and competition with other DNA sequences known to bind this protein suggest that this is the interaction detected.

These *in vitro* studies have identified protein DNA interactions which are reasonable candidates for regulation of the globin gene *in vivo*. For a more definitive assessment of these interactions, in particular interactions in the context of chromatin, we are developing an episomal vector system to study composition and structure of the regulated gene *in vivo*. The potential of this type system has been well demonstrated in studies from our laboratory in *S. cerevisiae*; we feel it is now time to extend such work to larger eukaryotic cells. The system under development uses an Epstein-Barr virus based vector. After transfection of the vector into K562 cells by electroporation and selection with hygromycin, Southern blot analysis of Hirt extracts showed individual clones bearing up to 50 copies of the vector. No integrated copies of the EBV sequences were present and transcription of the endogenous  $\epsilon$ -globin gene was unaffected by the episome. A marked  $\epsilon$ -globin gene is now being inserted into the vector for study of its regulation and structure.

### Developmentally Regulated Genes

Another set of mammalian developmentally regulated genes of interest are those coding for the three proteins of the murine zona pellucida, an extracellular glycoalyx which surrounds the growing oocyte, functions to mediate species specific sperm interactions, prevents polyspermy, and protects the embryo prior to blastocyst implantation. We have reported previously cloning of cDNA and

homeobox containing protein,  $\alpha 1$ , are necessary to repress the expression of haploid specific genes in  $a/\alpha$  diploid cells. We have now shown that these two proteins bind as a heterodimer to the operator of a haploid specific gene to block transcription. Coding sequences for  $\alpha 1$  and  $\alpha 2$  were fused to a SP6 promoter and RNA synthesized *in vitro*. This RNA was then translated in a wheat germ extract containing  $^{35}\text{S}$ -methionine to produce radiochemically pure proteins. Mobility shift assays showed that only cotranslated  $\alpha 1$  and  $\alpha 2$  were capable of binding to a synthetic oligonucleotide containing the haploid specific operator. A deletion of  $\alpha 2$  which retains DNA binding activity was used to unequivocally demonstrate that a single molecule of this protein was involved in the protein-DNA complex and two dimensional gel electrophoresis was employed to similarly demonstrate a 1:1 stoichiometry of  $\alpha 2$  and  $\alpha 1$  in the complex. We conclude that  $\alpha 1$  and  $\alpha 2$  bind to the haploid operator as a heterodimer,  $\alpha 1/\alpha 2$ ; this immediately suggests a mechanism for the dual regulatory activities of  $\alpha 2$  protein.

Others have mapped the DNA contacts for  $\alpha 2$  and MCM1 at an  $a$ -specific operator *in vitro*; MCM1 binds to the central region of the operator and the  $\alpha 2$  dimer binds to the peripheral regions on opposite faces of the DNA double helix. We used methylation interference to map contacts for the  $\alpha 1/\alpha 2$  heterodimer on a haploid specific operator. Contacts occur as two lobes which wrap most of the way around the double helix, leaving an irregular strip on one side of the helix where contacts apparently do not occur. The contact pattern and the stoichiometry exclude models where  $\alpha 1$  substitutes for MCM1 to alter the binding specificity of  $\alpha 2$  when it represses haploid specific genes. Demonstration that  $\alpha 1$  is present in the repressive complex excludes models where the function of that protein is to alter  $\alpha 2$  (by modification or proteolysis) and thereby change its specificity. We conclude that the different DNA binding patterns of the heterodimer and the homodimer/MCM1 complex explain the dual regulatory capacity of  $\alpha 2$ .

Homo- vs. heterodimer formation has recently been shown to be important in differential gene regulation by two other classes of *trans*-acting factors, leucine zipper proteins and the helix-loop-helix proteins. This is the first example of a similar situation for homeobox containing proteins, extending to a third group of factors the possibility of generation of several regulatory specificities in a combinatorial fashion.

We have previously shown that formation of a nucleosome over an initiation site can block transcription of RNA polymerase III genes *in vitro*; similarly, elongation by this polymerase was also inhibited by formation of nucleosomes. To determine whether elongation is also limited *in vivo*, we constructed derivatives of a tRNA gene which would yield 800 or 1100 nt transcripts in a multicopy yeast plasmid and introduced these into yeast. The former yields RNA of about 800 nt together with several smaller species while the latter only yields short RNA species. The chromatin organization of the plasmids remains to be determined as does the possibility of stop signals in the DNA per se by *in vitro* transcription of naked DNA in yeast extracts. A marked tRNA gene has also been placed next to sequences that have been shown, in other circumstances, to position a nucleosome (the ARS C domain and the  $\alpha 2$ /MCM1 binding site). Even though the predicted nucleosome position would place the tRNA start site in the middle of a nucleosome, there is no effect of these DNA changes on transcription of the tRNA gene *in vivo*. Studies of chromatin structure of the minichromosomes are in progress to determine whether the tRNA gene can be transcribed even when in a nucleosome or whether active transcription precludes formation of a positioned nucleosome.

One of the genes transcribed by polymerase III is that for 5S ribosomal RNA. We have studied the structure of yeast minichromosomes containing yeast 5S genes. The chromatin structure of the 5S gene reveals extensive protein-DNA interactions. A portion of the footprint on the gene is similar to that arising from the *in vitro* interactions of TFIIIA with the 5S DNA. Other proteins must bind to produce the total footprint, which extends towards the 5' end of the gene from the TFIIIA binding site. We have made multiple base substitution mutants in and near the TFIIIA binding site; this transcription factor is known to be essential for assembly of an active transcription complex. Analysis of RNA reveals no transcripts for the majority of the mutants. Nonetheless, the DNaseI footprints for all base substitution mutants are closely similar to that for the active, wild type gene. Recent studies of others *in vitro* using *Xenopus* extracts have similarly shown DNaseI footprints akin to wild type for point mutants of the TFIIIA binding region, but detectable changes with deletion mutants. We are therefore extending our yeast studies with deletion mutants in regions

recombination. A number of putative positive pools are being analyzed by limiting dilution; if successful, these clonal lines will be injected into blastocysts to create chimaeras to generate murine lines with a mutant ZP3 allele.

### Signal Transduction and Hormone Action

Studies of gene regulation during development of *Dictyostelium discoideum* provide a bridge between the nuclear studies outlined above and the studies of hormonal regulation detailed below. *Dictyostelium* is a cellular slime mold that grows as a unicellular amoeboid organism. If amino acids are depleted in the medium, the organism undergoes a well defined developmental program leading to a multicellular aggregate with two distinct cell types. Cyclic AMP is critical for several steps in development, serving as a chemoattractant and a hormone-like signalling molecule. Our interest is in signal transduction during development -- how environmental information is translated into cytoplasmic and nuclear adaptation by the cell. We have previously defined a number of genes whose temporal and spatial regulation appear to be under control of cAMP signalling. We now are studying the genes for proteins involved in signal transduction.

Work of others using binding kinetics, rates of desensitization and phosphorylation, molecular sizes and responses to divalent cations and guanine nucleotide analogs has suggested that two classes of cyclic AMP receptors (cARs) are present during early *Dictyostelium* development. One class is thought to be coupled to adenyl cyclase and the other to phospholipase C. Both classes cycle through a sensitive and a desensitized state in response to the cAMP oscillations which occur during early development. There was thought to be a possibility that another class of cAMP receptors was present following aggregation; these might respond to a continuous (non-oscillatory) cAMP stimulus and might regulate cell type differentiation and morphogenetic movement.

We have isolated and characterized four cAR genes which encode distinct cAMP receptor forms. Based on their deduced amino acid sequence, all four proteins appear to have seven trans-membrane domains, similar to other receptors that interact with G-protein transducers. The gene for cAR1 is expressed at very high levels during early development. This receptor protein has a carboxyl terminal intracellular domain which is rich in seryl residues; they may constitute a target for the extensive phosphorylation associated with receptor desensitization. It is likely that the cAR1 receptor has its effects through the adenyl cyclase second messenger pathway. When the normal pattern of cAR1 expression is disrupted by either antisense RNA or gene disruption, development is completely arrested.

The three other cAR genes are maximally expressed later in development and have distinct spatial patterns of expression in the multicellular aggregate. The carboxyl terminal intracellular domains of these three gene products lack the serine-rich character of cAR1; rather, they contain runs of basic amino acids. We would suggest that these three proteins might be responsive to continuous cAMP stimulation and required for cytodifferentiation and morphogenesis; disruption of their expression will be highly informative.

Thus, the multiple functions attributed to the cAMP receptor of *Dictyostelium* may be fulfilled by several receptor proteins encoded by different genes. In the context of multiple receptor genes with different temporal and spatial patterns of expression, we have begun to identify promoter and operator sites necessary for transcription of each gene to allow study of the regulatory elements responsible for their activity. A fifth candidate cAR gene has been identified as well as genes for several other receptors which interact with G-proteins.

Signal transduction has also long been an interest of members of LCDB who study the mechanisms whereby hormones regulate cellular metabolism, particularly in isolated adipocytes, a model for molecular endocrinology established by Dr. Martin Rodbell, a former member of the laboratory. Recently, improvements in the isolation of rat fat cells have led to a preparation which is highly reproducible in its metabolic characteristics and accurately mirrors the *in vivo* situation. Current interests involve the regulation of lipolysis by hormones which stimulate adenylate cyclase and by insulin. Of particular interest is the possible interaction between the insulin receptor and the enzymes

genomic clones for ZP3 (the sperm receptor protein) and ZP2, characterization of the timing of expression of the genes, their genomic organization, chromosomal localization, and predicted protein structure of the gene products. We have isolated and characterized the gene for human ZP3 and described its similarities to the murine gene. We have now isolated the human ZP2 gene and are actively involved in comparison of its structure and the predicted structure of the gene product with the mouse homologue.

Using poly dT and an oligonucleotide based on exon 1 of human ZP3, we have isolated a full length human ZP3 cDNA clone from ovarian RNA by PCR. The short 3' and 5' untranslated regions of the mouse RNA are conserved in the human message. Human ZP3 has a single open reading frame that encodes a 424 amino acid protein. Similarities in the size of the ZP3 transcripts among mouse, rat and rabbit, together with the similarities of mouse and human cDNA's suggests a common motif for all mammalian ZP3 messages.

The amino acid sequence of human ZP3, deduced from the cDNA, is 67% identical to mouse ZP3. Predictions of secondary structure indicate an even higher degree of homology; many of the differences are conservative substitutions. Similarities among the ZP3 proteins may be important in providing structural integrity to the zona pellucida and in positioning particular domains for sperm-egg interactions. While it is not known whether human ZP3 also functions as the species specific sperm receptor, it is likely that this is the case. Thus, the regions of maximal difference between the proteins of the two species may be of particular importance in species specific fertilization. Of the four regions of the human ZP3 protein that are most dissimilar to the mouse protein, the two most C-terminal are predicted to be hydrophilic domains that presumably reside on the protein's surface, suggesting a possible role in sperm recognition. The cloning of two putative sperm receptors, each in a species for which *in vitro* fertilization is possible, may provide the necessary reagents with which to gain additional information about the protein and carbohydrate domains that are critical for species specific fertilization.

Given our observations which demonstrate cross hybridization for the ZP genes among a number of mammals, we suggest that the signals which control oocyte specific expression of these genes might similarly be conserved. We therefore have isolated and characterized zona genes from a human genomic library for comparison with the murine genes. The human ZP3 gene is composed of eight exons, similar to mouse, but spanning 18.3 kbp, more than twice the size of the mouse gene. Four similar elements of eight to fifteen bp with 82-90% identity are present at nearly the same locations in the 250 bp immediately 5' to the human and murine ZP3 genes; closely related elements are present upstream of the ZP2 genes of both species. None of the sequences are identical to previously identified transcriptional regulatory factor binding domains.

To determine the functional significance of these DNA elements, the  $\beta$ -galactosidase or luciferase reporter gene has been placed under control of an 840 bp fragment from the 5'-flanking region of the mouse ZP3 gene and microinjected into growing oocytes, the single cell type which expresses ZP3. Cells expressed the reporter gene; controls showed that fibroblasts did not express the reporter, although both cell types expressed  $\beta$ -galactosidase under control of an RSV promoter. Deletion analysis has shown that oocyte specific expression requires only 470 bp of 5'-flanking sequence; this region of DNA contains the conserved elements described immediately above. Mutations in two of the putative *cis*-acting elements inhibit expression of the reporter gene in developing oocytes. We are currently determining whether oocyte specific proteins bind to these DNA elements and might therefore be candidates for mediators of developmental and tissue specific expression of these germ line genes.

We have begun experiments whose eventual goal is study of the role of oocyte development in ovarian morphogenesis; transgenic mice will be derived both by microinjection of paternal pronuclei and through the use of embryonic stem (ES) cell fusion into blastocysts. The prototype experiment involves gene disruption of one of the ZP3 alleles in ES cells to create a null mutation. A neomycin resistance cassette has been inserted into a 6.5 kbp fragment of mouse DNA containing the first five exons of the ZP3 locus. At the 3' terminus of the fragment, an HSV thymidine kinase gene was inserted, allowing simultaneous selection of insertion mutants with G418 and gancyclovir. DNA was isolated from pools of neomycin resistant, TK<sup>-</sup> cells and analyzed by PCR to detect homologous

which metabolize phospholipids to produce secondary signalling molecules; specifically, we have examined the question of insulin receptor action on hydrolysis of inositol phospholipids and the release of calcium-mobilizing metabolites.

The receptors for a number of hormones that, like insulin, contain intrinsic tyrosine kinase activity, initiate responses by activating an inositolphospholipid-specific phospholipase C (PLC) which hydrolyzes  $\text{PIP}_2$ , resulting in the release of the calcium-mobilizing metabolite,  $\text{IP}_3(1,4,5)$ . Many laboratories have failed in their attempts to demonstrate that insulin stimulates the above reaction. Previously, we demonstrated that insulin rapidly activates adipocyte protein kinase C, another reaction in which insulin was not thought to participate. We have now applied the cell manipulation techniques established for the protein kinase C studies to an investigation of insulin effects on inositol-containing phospholipids. Indeed, insulin at physiological concentrations stimulates the formation of  $\text{IP}_3(1,4,5)$ , an effect that peaks within 5-10 seconds. The inositol phosphate disappears within 20-30 sec, which probably accounts for the failure of others to detect this phenomenon. Concurrent with  $\text{IP}_3$  formation, we find that insulin rapidly (again, within seconds) stimulates both the disappearance or formation of several species of inositol-containing phospholipids, including some that differ from phospholipids known to be altered by other hormone receptors.

Comparative studies with vasopressin, known to stimulate  $\text{IP}_3(1,4,5)$  formation in adipocytes, produced surprising results. Vasopressin produced the spectrum of inositol phosphate metabolites one expects from PLC breakdown of  $\text{PIP}_2$ . In contrast, low level of such metabolites were found following exposure of cells to insulin, which stimulates the formation of a several unidentified inositol-containing water soluble products that were not seen with vasopressin. These results suggest that insulin might act through a hitherto unknown pathway, a speculation buttressed by the following observation. Other tyrosine kinase-containing receptors (e.g. EGF, PDGF) are thought to stimulate  $\text{PIP}_2$  breakdown by phosphorylating and activating the  $\gamma$  isoform of PLC. However, we find that physiological concentrations of insulin lead to rapid (5 sec) phosphorylation of the  $\beta$  form of PLC. Although the physiological significance of the above reactions is yet to be established, it is not unreasonable to suggest that these findings provide the foundation for identifying an immediate and important target for the insulin receptor.

Other efforts to study adipocyte metabolism, and the role of phosphorylation of specific proteins in same, have progressed during the past year. Although the surface of the lipid storage droplet in adipocytes is the site of both deposition and retrieval of stored lipid, little if anything is known of the molecular details of reactions which occur at this critical juncture. Previously, we discovered a complex of hormonally-regulated phosphoproteins (62-67 kDa) which we tentatively concluded were phosphorylation variants of a single, adipocyte-specific polypeptide that associates with the lipid storage droplet remnants in cell homogenates. Peptide analysis of four of the lipid associated phosphoprotein (LAPP) variants confirms that they are derived from a single polypeptide. With an affinity-purified antibody against one species (62 kDa), none of this protein is detected in a wide variety of tissues, providing further evidence that it is an adipocyte-specific protein, and the localization of the protein to the surface of the lipid storage droplet has been established by immunocytochemistry. Moreover, comparison of partial amino acid sequences with protein data bases reveals no significant homology with any known protein.

Collaboration between two LCDB working groups has led to significant advances in the research of each on several occasions. A particular example of the benefits of an interactive laboratory is the following interaction between the adipocyte group and the *Dictyostelium* signal transduction group. We have been using molecular markers, morphology and physiological state to monitor differentiation in the slime mold system. Culture of rat adipocytes and hormonal stimulation of differentiation of preadipocytes provide a similar system in a higher eukaryote for analysis of the effects of extracellular factors on differentiation. In particular, we have been interested in isolation and characterization of adipocyte-specific genes. Using the antibodies to LAPP62 discussed above and appropriate oligonucleotide probes based on the determined amino acid sequence of the protein, we have isolated several recombinant cDNAs which are predicted to encode overlapping portions of this adipocyte specific protein. Knowledge of the sequence of the protein and its homologues in other species will facilitate investigation of a molecule of putative importance in fat cell metabolism. Also of interest will be determination of the signals important for the expression of this and other genes

in adipocytes. Comparison with the promoter regions of oppositely regulated genes, the hormone sensitive lipase and glucose transporter, should be informative.

### Lipid Metabolism

In addition to the direct study of mechanisms of hormone action on fat cells, others in the laboratory study genetic defects in lipid metabolism, enzymes involved in lipolysis and clinical manifestations of lipid disorders. Mice born with combined lipase deficiency (*clد/clد*, a recessive mutation in the T/t complex of chromosome 17) develop extreme hyperchylomicronemia and die within three days if allowed to suckle. They have very low levels (<5% of normal) of lipoprotein and hepatic lipase activities (the genes for these glycoproteins are located on chromosomes 9 and 11, respectively), their tissues are virtually devoid of fat, and 95% are tailless. Studies *in vivo* showed that brown adipose and other tissues of the *clد/clد* mice synthesized normal sized lipoprotein lipase protein but the enzyme was inactive and not transferred to capillaries, the normal site of action of the enzyme. While the chromosomal localization of the genes and the mutation suggests that structural alterations of the primary structure of the lipases is not the root of the phenotype, this was suggested last year by Southern blot analysis of the lipoprotein lipase gene and recently demonstrated by RNase A protection analysis for the hepatic lipase gene.

We have studied the synthesis and secretion of both lipases in normal and *clد/clد* cultured brown adipocytes, making use of inhibitors of glycoprotein-processing enzymes to try to localize the step blocked in the secretion of the lipases in the mutant animals. Normal adipocytes synthesize active, secreted lipoprotein lipase with two endo H resistant oligosaccharide chains. Tunicamycin treatment leads to an inactive, unglycosylated lipase which is retained in the endoplasmic reticulum. Golgi mannosidase inhibitors lead to an active, secreted lipase but one with endo H sensitive oligosaccharide. Monensin leads to an active, but nonsecreted lipase which is retained in Golgi. We conclude that lipase becomes active and secretable at some stage(s) between core glycosylation in endoplasmic reticulum and mannosidase trimming in Golgi. The significance of processing lipoprotein lipase to the endo H resistant form remains a mystery.

Adipocytes from *clد/clد* mice synthesize inactive lipase with high mannose type oligosaccharides that is retained in endoplasmic reticulum. Normal lipase partitions between aqueous and Triton X-114 phases while mutant lipase is only in aqueous solution, suggesting that binding of lipase to membranes may be defective in *clد/clد* cells. Transport of certain proteins from endoplasmic reticulum is known to depend on binding to membranes.

The results of study of hepatic lipase in normal and mutant mice suggest that the situation is more complex than previously imagined. We have shown that rat hepatic lipase is localized in the extracellular space; it has long been known that plasma lipase is low in rats and increased by heparin injection which releases the enzyme from hepatocytes. In contrast, in mice, plasma lipase is high and not increased by heparin; the assumption would be that lipase is not in the extracellular space in significant amount in mice. Yet, in *clد/clد* mice, immunofluorescence localization showed hepatic lipase in the extracellular space, indicating secretion of this protein in contrast to lipoprotein lipase. Why hepatic lipase functions in the extracellular space of rats and apparently does not in the mutant mice remains an enigma.

We have recently cloned a cDNA for murine hepatic lipase. Predicted protein sequence is, overall, similar to that for rat and human enzymes. There are, however, significant differences in structure of two of the three predicted heparin binding domains, those for the mouse contain a higher concentration of acidic residues. This may account for the lower affinity of the mouse enzyme for heparin. It would be of interest to know if these differences also account for the different locations of the enzyme activity in the different species. We are beginning studies of the synthesis and secretion of the two lipases in normal and mutant hepatocytes in culture.

A possible role for lipoprotein lipase in the cachexia of tumor patients has been suggested by studies in LCDB in the past two years. Previously we found that the cytokine interleukin 6 (IL-6) inhibits lipoprotein lipase activity in isolated or cultured adipocytes, and it is known that injection of tumor necrosis factor (TNF or cachectin) leads to elevated plasma IL-6 concentrations. We now find that

resistance, an inability to store fat, and hyperlipidemia. Given the inhibition of adipocyte differentiation by various cytokines, we have examined sera from a number of lipoatrophic patients. Initial findings indicate that lipoatrophic, but not normal, subjects exhibit elevated serum interferon concentrations. Differences in fat distribution have been suggested to have major health effects in humans. In an attempt to determine if variations in plasma membrane hormone signalling systems account for variations in fat deposition patterns, we have initiated studies on the receptor populations, G-protein content, and adenyl cyclase activity in adipocytes isolated from different regions (abdominal/gluteal) of normal and obese patients both before and after a weight reduction program. By applying techniques established for isolating rat adipocytes, we have succeeded in isolating human adipose cells and preparing membranes that exhibit highly reproducible profiles of hormone responsiveness for any given donor.

Studies of dihydrofolate reductase, the target enzyme for methotrexate, an antimetabolite of high clinical importance in treatment of cancer and autoimmune diseases, have been carried out in LCDB for more than two decades. Collaborative investigations of drug derivative interactions with the enzyme from several vertebrate sources continue. Quantitative structure-activity relationships have been determined for the chicken liver and bacterial enzymes interacting with a series of 5-(substituted benzyl)-2,4-diaminopyrimidines. Steric bulk was found to be of major significance in binding to the bacterial enzyme while hydrophobicity was of primary importance for the vertebrate protein.

Studies of barnase activity have been facilitated by development of a novel assay using a fluorescent substrate, polyethenoadenosine. Four kinetic steps are observed in hydrolysis, the first three being hydrolysis to oligomers, dimers and finally monomers; these all involve transesterification to 2'-3' cyclic phosphates. The last step, slower than the others, is hydrolysis of the cyclic monomer. A systematic examination of the effects of pH, ionic strength and substrate concentration has now been carried out. The pH dependence of hydrolysis of dinucleotides differs markedly from that for larger substrates and the rate of hydrolysis is much lower for the dinucleotides. This suggests that modelling of substrate binding to barnase should use longer substrate analogs than the dinucleotides typically used for such studies with other ribonucleases.

adipocytes isolated from animals receiving TNF possess greatly reduced lipoprotein lipase activity, providing further evidence of a role for IL-6 in cachexia. We also find that TNF stimulates IL-6 production by adipocytes, suggesting an autocrine role for IL-6 in fat tissue. Moreover, the adipocyte precursor cells, 3T3-L1 fibroblasts, produce high levels of IL-6, but production drops precipitously when these cells are exposed to agents (dexamethasone, insulin) that stimulate differentiation into adipocytes. These results suggest that the absence of cytokines may be necessary for lipid storage, and provide an explanation for reduced lipoprotein lipase activity induced by both IL-6 and TNF.

We also study lipid metabolism in human diseases such as type C Niemann-Pick (NP-C) disease, an autosomal recessive neurovisceral lipid storage disorder. In normal cells, cholesterol derived from endogenous biosynthesis and exogenous sources such as low density lipoprotein and non-lipoprotein cholesterol translocates to the Golgi. A sphingolipid precursor analogue, a fluorescent ceramide, also accumulates in Golgi, targeted to that site by cholesterol. Cultured cells derived from patients with NP-C disease accumulate cholesterol in both Golgi and lysosomes. Liver biopsies from NP-C patients contain large amounts of cholesterol and other lipids in sinusoidal cells and hepatocytes. Enzyme and immunochemical methods show lipid accumulation in both Golgi and lysosomal compartments, perhaps reflecting defective intracellular transport of endogenous and exogenous lipids. Having shown that the Golgi plays a role in such lipid transport, we speculate that the lipid storage disorder in NP-C disease may reflect a lesion in this function of the Golgi.

### Biotechnology and Applied Biochemistry

Several areas of research in LCDB are closer to bringing the results of basic investigations to use in clinical situations or for production of materials for other research. I have grouped them together in this section of the report, even though their subject matter is not logically coherent.

The Biotechnology Unit is a service facility for all of NIH in addition to serving in research and development of biotechnological methodology, particularly production of microbial, fungal and tissue culture cells and initial large scale processing of biologicals. During the past year over 120 fermentations in volumes from 10 to 300 liters were performed. Mammalian tissue culture cells were grown for various groups at NIH in volumes up to 50 liters. A number of fermentations were for production of toxins for potential clinical applications; among these were growth of *Pseudomonas aeruginosa* for isolation of exotoxin to be used in development of antineoplastic directed cytotoxic agents and growth of *Vibrio cholera* for toxin production for vaccine development. Other studies concentrated on improvement of yields of toxins and biomass; yields of bacterial cells up to 100 g/L have been obtained recently. A recovery and purification process for production of gram quantities of recombinant mutant exotoxin A from *E. coli* was devised. The previously developed scheme for production of cholera toxin was modified for optimal preparation of the b subunit of the toxin; yields of up to 30 mg/L were obtained.

Noted before were studies of ZP3, the murine sperm receptor. We had shown previously that antibody to ZP3 could be used to passively immunize mice against conception in an effective, but reversible, fashion and last year reported active contraceptive immunization of mice with a sixteen amino acid ZP3 peptide coupled to KLH. The peptide contained an epitope defined in our early studies of monoclonal antibodies to zona proteins. While our earlier studies showed no ovarian histopathology in immunized NIH Swiss mice, more recent work has determined that in mouse strains known to be susceptible to ovarian autoimmune disease (B6A, BalbC), immunization with this peptide does result in significant ovarian pathology. A T-cell epitope on the peptide has been identified; it is distinct from the seven amino acid B-cell epitope. We are now determining if the B-cell epitope alone is sufficient for immunocontraception without causing ovarian damage. We have also defined a ZP2 B-cell epitope and are studying the efficacy of this as an immunogen, both alone and in combination with the ZP3 epitope. Given the close relatedness of the zona genes from a variety of mammalian species, the potential to extend such studies to other animals is high, particularly as we gain expertise in definition of specific immunogenic regions of the zona proteins.

Studies on adipocytes, hormones and cytokines are being extended to investigations using human tissues. Lipoatrophic diabetes is a poorly understood metabolic disorder characterized by insulin-



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15005-15 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Regulation of Adipocyte Metabolism

PRINCIPAL INVESTIGATOR (Last other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	Constantine Londos	Research Chemist	LCDB:NIDDK
Others:	John J. Egan	Staff Fellow	LCDB:NIDDK
	Andrew S. Greenberg	Senior Staff Fellow	LCDB:NIDDK
	Nira B. Garty	Visiting Fellow	LCDB:NIDDK
	Sheree A. Wek	Biologist	LCDB:NIDDK

## COOPERATING UNITS (if any)

A.R. Kimmel, J. Blanchette-Mackie, R.O. Scow, C. Mateo, LCDB:NIDDK; M.A. Moos, K.B. Seamon, CDB:DB; R.P. Nordan, D.M. Jablons, DCT:NCI; J.C. Calvo, LTPD:CH; P. Coon, A.P. Goldberg, U. Maryland, Baltimore

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Membrane Regulation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

3.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Three areas of research on hormonal control of adipocyte metabolism are summarized: (A) Previously, we reported the discovery of several species of hormonally regulated, lipid storage droplet-associated phosphoproteins (LAPP). Protease digestion and peptide mapping of four LAPPs confirms that they are phosphorylation variants of a single polypeptide. Western blot scans of rat tissues with an affinity-purified antibody raised against LAPP indicates that it is adipose cell-specific. Finally, immunocytochemical studies with 3T3-L1 adipocytes reveals that LAPP is tightly associated with the lipid droplet surface in intact cells. (B) Extending our finding that insulin rapidly activates protein kinase C activity in adipocytes, we find that insulin also stimulates the breakdown of the an inositol phospholipid, leading to release of IP3(1,4,5), the calcium-releasing metabolite. IP3 formation peaks within seconds of exposure of cells to physiological insulin concentrations, concomitant with increased phosphorylation of a specific isoform of phospholipase C, a potential target for the insulin receptor tyrosine kinase activity. Interestingly, comparative studies of inositol phosphate metabolites formed with insulin and vasopressin indicate that insulin-stimulated hydrolysis of inositolphospholipids may proceed through a hitherto undescribed pathway. (C) Cachectic patients exhibit high concentrations of plasma Interleukin-6 (IL-6), and we have found that IL-6 acts directly on both freshly isolated adipocytes and on cultured 3T3-L1 adipocytes to decrease lipoprotein lipase (LPL) activity. We now find that adipocytes isolated from animals administered IL-6 possess greatly reduced LPL activity, providing further support for the notion that IL-6 may play a role in cachexia. A potentially related finding is that factors that stimulate the differentiation of precursor 3T3-L1 fibroblasts into adipocytes strongly inhibit IL-6 production by the precursor cells. Finally, on the matter of cytokine effects on adipose cells, we find elevated levels of interferons in sera of subjects with lipodystrophy, a syndrome characterized by an inability to store fat. Taken together, these observations strongly suggest a pathophysiological role for cytokines in a number of diseases manifested by abnormal lipid storage.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15004-15 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Modulation of Hormone-Responsive Systems by ras Oncogene Product

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Michael C. Lin  
Others: Beatrix H. WhiteResearch Chemists  
IRTA FellowLCDB:NIDDK  
LCDB:NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project was terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15102-30 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of a ribonuclease and its inhibitor from Bacillus amyloliquefaciens

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: Robert W. Hartley

Research Physicist

LCDB:NIDDK

Others: Peter FitzGerald

Senior Staff Fellow

LCDB:NIDDK

## COOPERATING UNITS (if any)

Dr. Yves Maugen, Laboratoire de Physique, Centre Pharmaceutique, Universite de Paris-Sud. Dr. Jean Garnier, Protein Engineering Unit, Biotechnology INRA, Jouy-en-Josas, France. Dr. Guy Dodson, Chem. Dept., University of York (foreign)

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are used as a model system for the study of protein folding and protein-protein interactions. Barnase is one of a homologous group of ribonucleases occurring in both prokaryotes and eukaryotes.

Recombinant DNA techniques are being applied with three major aims: (1) to facilitate production of wild type and mutant proteins; (2) to examine the structural and control sequences of the genes; and (3) to make specific changes in the sequences to test theories of folding and probe the barnase-barstar interaction.

The lethal effect of the cloned wild type barnase gene can be repressed by expression of the barstar gene on the same plasmid. E. coli plasmid vectors have been devised for both proteins and both can now be obtained essentially pure in 100 mg quantities. DNA and amino acid sequences are known for both and the x-ray structure of barnase has been refined to 2.0 Å. The structures of both proteins in solution are being studied by 2-D NMR. A synthetic fluorescent substrate has been used to study hydrolysis kinetics and to look at the kinetics and stability of the barnase-barstar interaction for native and mutant proteins. Some 50 variations in the sequence of each protein have been obtained by oligonucleotide-directed mutagenesis. Some of these were aimed at specific questions, but most are part of a survey of the protein surfaces designed to locate their areas of interaction. Our most important finding with these mutants is that the two Cys residues of barstar can both be replaced by Ala without loss of activity or yield, greatly simplifying future studies of barstar folding.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15100-20 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein Nucleic Acid Interactions: Chromatin Structure and Function

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	R.T. Simpson	Laboratory Chief	LCDB:NIDDK
Others:	A. Dranginis	Senior Staff Fellow	LCDB:NIDDK
	R. Morse	Senior Staff Fellow	LCDB:NIDDK
	R. Parker	Senior Staff Fellow	LCDB:NIDDK
	S. Y. Roth	Senior Staff Fellow	LCDB:NIDDK
	M. Shimizu	Visiting Fellow	LCDB:NIDDK
	C. Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK

## COOPERATING UNITS (if any)

T. Richmond, ETH, Zurich, Switzerland (foreign)

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.5

## PROFESSIONAL:

6.5

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
 ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project uses a variety of approaches to address fundamental questions relating to chromatin structure, transcription and replication. We previously reported that the yeast  $\alpha 2$  protein organized chromatin structure; positioned nucleosomes flanked the binding site for this protein in a minichromosome. Using primer extension, we have now mapped these nucleosomes at base level resolution with the finding that the positioning is exquisitely accurate. Identical positioning is observed for the single genomic copy of a gene repressed by  $\alpha 2$ ; since the sequences flanking the operator differ completely for the two situations, this suggests an active organization of chromatin by the repressor. We have addressed the possible mechanism of positioning with the finding that deletions of amino acids 4-19 or 4-23 of histone H4 abolish positioning around the  $\alpha 2$  operator, suggesting a direct interaction of the repressor with nucleosomal histones. Alpha2 also functions to repress the expression of haploid specific genes in yeast diploid cells; another homeobox containing protein,  $\alpha 1$ , is required for this activity. We have conclusively demonstrated that a heterodimer,  $\alpha 2/\alpha 1$ , is the structural entity which binds to haploid specific operators to effect this repression. Mapping of DNA contacts for the heterodimer shows a markedly different pattern from that for the  $\alpha 2$  homodimer + MCM1 dimer that represses  $\alpha$ -specific genes. The data explain the dual repressive activities of the  $\alpha 2$  protein. This is the first demonstration of differential function of homo- and heterodimers of homeobox containing proteins and suggests the possible importance of such dimers in combinatorial regulation of genes in larger organisms. Studies of the chromatin structure and transcription of RNA polymerase III genes in yeast are continuing in attempts to correlate nucleosome positioning and interactions of regulatory factors with transcriptional initiation and elongation. A region replete with DNA sequences similar or identical to those bound by known transcriptional regulatory factors, 1 kb 5' to a heat shock gene of yeast, seems likely to function in regulation of transcription of the gene. Deletion of less than 150 bp in the putative regulatory region leads to a phenotype similar to deletion of the entire structural gene. Collaborative studies of high resolution core particle structure and higher order chromatin structure are continuing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 15400-16 LCDB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormones, Lipoprotein Lipase and Lipid Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Robert O. Scow Chief, Endocrinology Section LCDB:NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Project was terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Folic Acid (Dihydrofolate Reductase)

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Bernard T. Kaufman

Research Chemist

LCDB:NIDDK

Others: John Bieri

Scientist Emeritus

LCDB:NIDDK

## COOPERATING UNITS (if any)

Department of Chemistry, Pomona College, Claremont, CA  
Nutrition Research Center, USDA, Beltsville, MD

## LAB/BRANCH

Laboratory of Cellular &amp; Developmental Biology

## SECTION

Nutritional Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dihydrofolate reductase (DHFR) is a ubiquitous enzyme that is the target for the antifolate drugs. The antifolates are of continuing importance not only in neoplastic disease, but also of increasing utility in autoimmune disorders, AIDS-related opportunistic complications (including parasitic and bacterial infections) and such infections in general. Problems of extensive toxicity and lack of selectivity of the currently available drugs such as methotrexate (MTX) and trimethoprim (TMP) provide the impetus for further study. The correlation techniques of QSAR (quantitative structural-activity relationships) are such an approach to gain understanding of the forces that mediate the interaction of ligands (drugs) with their targets, thus suggesting the minimum number of structural variations in a base compound to achieve an inhibitor that is uniquely selective and potent. DHFR is a unique system to analyze via QSAR since not only are the X-ray crystallographic structures well defined for the reductases from chicken liver, E. coli and L. casei, but also hundreds of closely related antifolate inhibitors have been synthesized.

Using methods developed in this laboratory, DHFR from several animal sources have been extensively purified and are available for investigation of enzymatic properties, mechanism of action, and interaction with metabolic inhibitors and drugs, in collaborative studies.

The inhibition of chicken liver DHFR by a series of 5- (substituted benzyl)-2, 4-diaminopyrimidines was analyzed in terms of parameters relating to substituents on the aromatic side chains of the inhibitor, including hydrophobicity, molar refractivity, the Hammett constant, certain indicator variables and polar and non-polar interactions. While the QSAR results appear to be in general agreement with X-ray crystallographic models at 2.8-Å resolution, prediction of specific structural details is not yet possible.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15404-06 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ultrastructural Immunocytochemistry of Lipid Metabolism in Cultured Cells and Tissues

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: E. Joan Blanchette-Mackie

Research Biologist

LCDB:NIDDK

Others: Robert O. Scow  
Nancy K. DwyerChief, Endocrinology Sec.  
BiologistLCDB:NIDDK  
LCDB:NIDDK

## COOPERATING UNITS (if any)

Dr. Richard E. Pagano, Dept. Embryol., Carnegie Inst. of Washington, Baltimore, MD; Dr. Carl Alving, Dept. Membrane Biol., Walter Reed Army Inst. of Res., Washington, D.C.; Dr. Peter Pentchev, Dev. Metab. Neurol. Branch, NINDS, NIH; and Dr. Howard S. Kruth, Lab Exptl. Ather., NIH, NIH.

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Endocrinology Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

1

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hepatic lipase hydrolyses triacylglycerol in chylomicrons and very low density lipoprotein remnants in the liver. We have shown that hepatic lipase is present extracellularly in liver from normal rats and newborn mutant mice with a combined lipase deficiency (cld/cld). Combined lipase deficiency in mice is characterized by marked functional deficiencies of both lipoprotein lipase and hepatic lipase. Hepatic lipase is present beneath the sinusoidal endothelial lining cells and hepatocyte basal surface in the "space of Disse". In cld/cld mice lipid particles the size of chylomicrons and VLDL are also present in the "space of Disse". Hepatic lipase in the extracellular space is associated with matrix material, collagen and lipid particles. It appears that the extracellular matrix is a repository for hepatic lipase as well as lipoprotein particles and probably the site of hepatic lipase hydrolytic activity in normal rats. In contrast, the extracellular hepatic lipase in liver of cld/cld mice is hydrolytically inactive.

Cholesterol derived from endogenous biosynthesis and exogenous sources (LDL-cholesterol and non-lipoprotein cholesterol) translocates to the Golgi. Cholesterol also plays a role in targeting fluorescent ceramide (C6-NBD-Cer), a precursor of sphingolipid, to the Golgi. Cholesterol accumulates in both lysosomes and Golgi in cultured cells derived from Niemann-Pick type C patients. We found Golgi involvement in the pathogenesis of the Niemann-Pick type C lipid storage disease. Liver biopsies from NP-C patients contain massive accumulations of cholesterol and other lipids in both hepatocytes and sinusoidal cells. Enzyme and immunocytochemistry revealed that lipid accumulation in lysosomes and Golgi compartments in liver cells probably reflects defective intracellular transport of both endogenously and exogenously derived lipids. The Golgi plays a role in intracellular lipid transport and the NP-C lipid storage disorder may reflect a lesion in this Golgi function.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 15401-18 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis and Transport of Lipoprotein and Hepatic Lipases in Tissues and Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Robert O. Scow

Chief, Endocrinology Sect.

LCDB, NIDDK

Others: Carmen Mateo

Visiting Fellow

LCDB, NIDDK

Charles J. Schultz

Guest Worker

LCDB, NIDDK

E. Joan Blanchette-Mackie

Research Biologist

LCDB, NIDDK

Albert E. Spaeth

Chemist

LCDB, NIDDK

## COOPERATING UNITS (if any)

Dr. Calvin Roff, DMN, NINDS, NIH; Dr. Kazuhiro Oka, Medlantic Research Foundation, Washington, D.C.; Dr. Leslie P. Kozak and Dr. Ulrike C. Kozak, Jackson Laboratory, Bar Harbor, Maine

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Endocrinology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

1.5

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mice born with combined lipase deficiency (cld/cld) have very low levels of lipoprotein and hepatic lipase activities, develop severe hypertriglyceridemia, and die within 3 days. The recessive mutation (cld) causing the deficiency is located on chromosome 17, whereas structural genes for these lipases are located on chromosomes 8 and 11, respectively. These lipases, both glycoproteins, are synthesized in parenchymal cells and transferred to endothelial cells where they act. Southern blot analyses of endonuclease-digests of the lipoprotein lipase gene and RNase A protection assays of hepatic lipase mRNA suggest that the structural genes for the two lipases are normal in cld/cld mice. Lipoprotein lipase synthesized in cld/cld brown adipocytes, unlike that in unaffected cells, is inactive, endo H-sensitive, and retained in endoplasmic reticulum. Lipoprotein lipase of normal cells is found in both aqueous and Triton X-114 extracts of cells, whereas lipase of cld/cld cells is found only in the aqueous extract, suggesting defective binding of the lipase to membranes, possibly endoplasmic reticulum, in cld/cld cells. Hepatic lipase in liver of cld/cld mice, although inactive, is secreted to the extracellular space. These findings suggest that the cld mutation affects the two lipases in different ways.

Unaffected mouse brown adipocytes synthesize lipoprotein lipase which is active, has endo H-resistant (complex) oligosaccharides, and is secreted. Cells treated with tunicamycin produce unglycosylated lipoprotein lipase which is inactive and retained in endoplasmic reticulum. Blocking the action of Golgi mannosidase I with 1-deoxymannojirimycin, or mannosidase II with swainsonine, causes formation of lipase which is totally endo H-sensitive, yet active and secreted. Monensin treatment also blocks processing of lipoprotein lipase to the endo H-resistant form without affecting the synthesis of active lipase. Secretion of lipase, however, is impaired because of the direct blocking action of monensin on transport between cis and trans Golgi. The findings indicate that lipoprotein lipase becomes active (dimeric) and secretable during some process(es) between core-glycosylation in endoplasmic reticulum and trimming by mannosidases in Golgi. The importance of processing lipoprotein lipase to the endo H-resistant form is not yet known.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15503-09 LCDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (60 characters or less. Title must fill on one line between the borders.)

## Regulation of Developmental Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Alan R. Kimmel  
 Others: Charles L. Saxe, III  
 John Louis  
 Gail Ginsburg

Research Chemist  
 Senior Staff Fellow  
 Staff Fellow  
 Biologist

LCDB:NIDDK  
 LCDB:NIDDK  
 LCDB:NIDDK  
 LCDB:NIDDK

## COOPERATING UNITS (if any)

Dr. Peter Devreotes, The Johns Hopkins Medical School  
 Dr. Andrew S. Greenberg and Dr. Constantine Londos, LCDB:NIDDK

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

How extracellular (hormonal, sensory or neuronal) signals regulate differentiation is a central question of cellular and developmental biology. We are interested in understanding how events at the cell surface ultimately manifest alterations in gene activity and cell fate. Dictyostelium has proven an excellent system for studying such mechanisms, lending itself to molecular, cellular, biochemical and genetic manipulation. Several aspects are being explored. We have continued our molecular analysis of genes which encode proteins required for signal transduction. Primarily, this has involved studies on the function and expression of a number of G-protein linked, cell surface receptors. We have isolated four receptor genes; each exhibits a distinct pattern of temporal and spatial expression during the Dictyostelium developmental cycle. Structural and functional analyses suggest that they couple to different effector systems. We have also tentatively identified several other genes which may encode additional receptors that interact with G-proteins. In a complementary approach, we have begun an analysis of hormonal regulation of mammalian differentiation, focusing on the isolation, regulation and function of genes expressed specifically in adipocytes. We have isolated several cDNAs which appear to be derived from mRNAs which encode a fat-associated protein preferentially expressed in adipocytes. Other adipocyte proteins of interest include hormone sensitive glucose transporter, lipoprotein lipase and hormone sensitive lipase. Preliminary results indicate that the genes for these proteins are differentially regulated in cultured adipocytes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Large-Scale Processing of Biological Material

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Joseph Shiloach  
Others: Jeanne B. Kaufman  
Michelle van de Walle

Research Chemist  
Biologist  
Guest Researcher

LCDB:NIDDK  
LCDB:NIDDK  
LCDB:NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The Pilot Plant (Biotechnology Unit) combines several different types of activities. It is responsible for the large-scale production of bacteria, mammalian cells and biologically active compounds from various sources. Parallel to this activity, it conducts process development work associated with these preparations in order to be able to execute them efficiently. In addition, the unit carries on research work not necessarily associated with a current project, but work that has long-term implications for the unit's performance.

During the last year, the unit carried out 123 different large-scale preparations, including micro-organism growth in volumes from 10 to 300 liters, mammalian cell growth up to 50 liter volumes and processing of various biological materials.

Development work was done in the growth optimization of various recombinant E. coli. An example is growth and recovery process for obtaining gr quantities of purified non-toxic exotoxin A lacking glutamic acid at position 553.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 15508-03 LCDB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure in Regulation of Mammalian Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Ann Dean	Research Chemist	LCDB:NIDDK
Others:	Qihui Gong	Visiting Scientist	LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. Lei L. Chen, Division of Hematology and Oncology, Department of Medicine, University of Connecticut Health Center, Farmington, CT

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are interested in how the temporal and tissue specific expression of a gene is brought about during development. The orderly activation and repression of individual members of the human globin gene family is a prominent example of this phenomenon. We are studying the changes in chromatin structure that these genes undergo when they are activated. We have used DNase I footprinting and the electrophoretic mobility shift assay to detect, in vitro, interactions between nuclear proteins from erythroid and non-erythroid cells and DNA sequences from regulatory regions both 5' and 3' to the human epsilon-globin gene. At least one of the interactions we observed was erythroid specific and corresponded to binding of EryF1/NF-E1/GF-1 to two sites in the promoter region. This protein also has strong binding sites in the region 3' to the gene. Other interactions are in the process of being analyzed. To investigate the functional significance of these protein-DNA interactions transient assays in K562 cells and HeLa cells are being employed in which transcription from the epsilon-globin promoter is monitored via a chloramphenicol acetyltransferase reporter gene. An episomal vector system is being developed in order to study in vivo the effect of these protein-DNA interactions on higher order chromatin structure and transcription of the epsilon-globin gene. Preliminary studies suggest that an EB virus-based vector can be maintained as an amplified episome in K562 cells. A marked epsilon-globin gene is being inserted into the vector and its regulation will be studied.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Gene Expression in Early Mammalian Development

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Jurrien Dean	Senior Investigator	LCDB:NIDDK
Others:	Li-Fang Liang	IRTA Fellow	LCDB:NIDDK
	Sarah Millar	Visiting Fellow	LCDB:NIDDK
	Dwayne Lunsford	Staff Fellow	LCDB:NIDDK
	Anne Baur	Chemist	LCDB:NIDDK
	Carol Kasten-Sportes	Guest Worker	LCDB:NIDDK
	Eric Lader	IRTA Fellow	LCDB:NIDDK

## COOPERATING UNITS (if any)

Frank Robey, LCDO, NIDR; Tom Fuerst, Molecular Vaccines, Inc, Rockville, MD; Kenneth Tung, Washington University, St. Louis, MO; Ralph Brinster, University of Pennsylvania, Philadelphia, PA

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.6

## PROFESSIONAL:

5.6

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mammalian zona pellucida is an extracellular matrix comprised of three sulfated glycoproteins (ZP1, ZP2 and ZP3) that surrounds growing oocytes, ovulated eggs and embryos. In mouse, ZP3 is the primary sperm receptor and induces the sperm acrosome reaction; ZP2 is a secondary sperm receptor; and both proteins play a role in the post-fertilization block to polyspermy. The genetic loci of mouse and human Zp-2 and Zp-3 have been characterized. The single copy mouse Zp-2 gene is located on mouse chromosome 7 and contains 18 exons (45-190 bp) spanning 12.1 kb. The human homologue is similarly arrayed. The mouse and human Zp-3 genes are each composed of 8 exons (92-338 bp) that span 8.6 and 18.3 kb of DNA, respectively. Mouse Zp-2 and Zp-3 are transcribed uniquely in oocytes and transcripts accumulate during the narrow two-week growth phase of oogenesis. Deadenylation and degradation of zona transcripts during meiotic maturation is associated with the cessation of zona protein synthesis in ovulated eggs. By comparing the 5' flanking sequences of the four cloned zona genes (human Zp-2 and Zp-3, mouse Zp-2 and Zp-3), conserved elements (7-12 bp) have been identified within the first 250 bp upstream of the transcription start sites. Reporter genes containing 470 bp of 5' flanking sequence from mouse Zp-3 are expressed after micro-injection into growing oocytes. Preliminary studies indicate that mutations of two of the defined DNA elements results in marked diminution of reporter gene activity. Studies are currently underway to identify trans-acting factors that bind to these motifs and modulate oocyte-specific gene expression. The primary amino acid sequence of mouse ZP2 and ZP3 (as well as human ZP3) have been deduced from full length cDNA clones of the cognate transcripts. Epitope libraries have been screened with anti-ZP2 and anti-ZP3 monoclonal antibodies, previously shown to act as effective contraceptive agents. Vaccination with the defined ZP3 epitope can result in long term, reversible contraception. Because the zona genes are conserved from mouse to man, this contraceptive strategy of vaccinating with "self" zona pellucida may be widely applicable among mammals.

## Polysaccharides in Morphogenesis

Two chitin synthetases, Chs1 and Chs2, are known to be present in yeast. Chs2 is essential for septum formation and cell division. The previous hypothesis, that Chs1 has a repair function during cell separation, has been now confirmed by unambiguous genetic evidence. The genes coding for the two chitin synthetases, CHS1 and CHS2, share an extensive homologous region but differ in the amino terminal sequence. By gene deletions and ligations we have determined that the amino terminal region of CHS2 is not required for either enzymatic activity or function.

Genetic analysis has confirmed that proteinase B is not required for activation of the Chs1 zymogen in vivo. Chs1 has been found to be more sensitive than Chs2 to certain antifungal agents.

## Enzymatic Basis of Detoxication

Among the thirty or so enzymes of detoxication is the group of esterases which are ubiquitously distributed among cells, with about 95 per cent of total activity present in the microsomal fraction of liver. The esterases fall into two classes based on catalytic mechanism. The major activity by far is with the serine-type esterases whereas a minor fraction has a different, as yet unknown, hydrolytic mechanism. An investigation of those enzyme that are in rat liver cytosol has resulted in the isolation of two electrophoretically homogeneous proteins that have a high affinity for aspirin and probably account for most of the hydrolysis of that ester in liver. These are serine-esterases which also catalyze the hydrolysis of a large variety of other esters.

A systematic examination of the second group, non-serine esterases, in the cytosol allowed the isolation of twelve esterases of varying molecular size and isoelectric point, all relatively uninhibited by paraoxon, but able to accommodate a very wide range of substrates with an overlapping specificity. Four of these enzymes were prepared in homogenous form and antibody was obtained against three of them; each antibody reacted only with the esterase that served as antigen and not with any of the other eleven esterases. In addition to the oxygen esters, thiol esters served as substrates but amides and peptides did not.

## II. PROTEIN SORTING AND TRANSPORT FUNCTIONS

Central to modern biology is the nature of the mechanism for the movement of macromolecules, glycoproteins in particular, not only into and out of the cell but also into specific organelles. Plural mechanisms are being sought from the viewpoint of the disciplines of somatic cell genetics, molecular biology, carbohydrate chemistry, endocrinology and biochemistry. The implications of the work extend from cell biology to applications in thyroid pathobiology and approaches to the AIDS virus.

## Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

An approach to dissecting the processes of endocytosis, glycoprotein biosynthesis and sorting is through isolation and analysis of mutants. Based on observations of aberrant compartmentalization of the M6P/IGFII receptor in a temperature-sensitive End1 mutant, perturbation of the intracellular pathways followed by this receptor was attempted. The results may be summarized as follows: 1. In the presence of weak base human diploid fibroblasts accumulate up to 40% of receptor-enzyme complexes in dense lysosomes; this accumulation represents a steady state and is rapidly reversed upon re-acidification of organelles. 2. Inhibition of protein synthesis effects rerouting of lysosomal enzymes present in the secretory pathway from a predominantly intracellular to a predominantly endocytic route of delivery to lysosomes. 3. Inhibition of protein synthesis also blocks accumulation of receptor-ligand complexes in lysosomes and effects a futile cycle whereby these complexes shuttle between the cell surface and an intracellular compartment(s). It is hypothesized that a rapidly turning-over protein is required for delivery of receptor-enzyme complexes to the late endosome via both the endocytic and intracellular pathways.

Electron microscopic examination of endocytosed tracers in a CHO cell mutant isolated for its accumulation of ligand in non-acidic vacuoles indicates that the mutant may be impaired in vesicular fusion.

## The Role of the Carbohydrate Moiety of Glycoproteins in Cellular Activity

The hepatic receptor for asialoglycoproteins was found to be modulated by the glucose concentration in the medium of the human hepatoma cell line, HepG2. The surface binding of asialoorosomucoid increased from 20 ng/mg of cellular protein to about 40 ng/mg as the glucose concentration was increased from 10 to 50 mg %. Scatchard plot analysis indicated a rise in the number of binding sites as well as a two-fold increase in binding affinity. The binding of antibody against the human receptor did not change, confirming that the actual number of receptors remained constant in face of an increased number of binding sites. Specificity of the glucose effect was indicated by the binding of insulin and transferrin to their respective receptors, which was unaffected under the conditions that increased asialoglycoprotein binding. The up-modulating effect of glucose was abolished by 2-deoxyglucose, an inhibitor of glucose metabolism, and by cycloheximide, an inhibitor of protein synthesis. The mechanism responsible for the modulation is currently unknown, although presumptive evidence is available to suggest that it is mediated by changes in the level of cyclic GMP.

## The Role of Intracellular Traffic in HIV Infection

CD4, the T-cell receptor for the human immunodeficiency virus, depends upon glycosylation for proper surface expression. Initial studies employing acute lymphoblastic leukemic cells were extended by the successful transfection of a plasmid containing the cDNA for CD4 for mutant and wild type Chinese hamster ovary cells. Characterization of these clones suggests that CD4 contains biantennary unsialylated complex-type oligosaccharides. The data also suggest that the inability of CD4 to reach the cell surface when it lacks carbohydrate is due to the selective degradation of unglycosylated CD4.



ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY AND METABOLISM  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory conducts research in several apparently disparate areas that include morphogenesis, differentiation, endocytosis, endocrinology, membrane transport, detoxication, and the physical and chemical behavior of proteins and nucleic acids. It does so by applying a broad array of different approaches. Resolution is being attempted by methods that stem from enzymology, biophysics, carbohydrate chemistry, cell biology and molecular biology. Although seemingly diverse, there is a common element to each of the subjects summarized here that is appropriate to the Laboratories' designation: biochemical, metabolic and physical approaches are being brought to bear on major problems encompassed by the Institute's charge. It is the close proximity of experienced investigators from diverse scientific disciplines, able to discuss their individual problems with each other, that provide synergistic effects for the resolution of the questions under investigation.

I. ENZYMES: FUNCTIONAL AND ABNORMAL

Several groups are active in this broadly designated area which covers the discovery of the several lesions in the gene for an enzyme whose absence leads to Tay-Sachs disease; the means by which enzymes play a role in morphogenesis; and the enzymatic mechanism by which aspirin is metabolized and detoxified.

The Genetic Lesions of Tay-Sachs Disease

Tay-Sachs disease is a recessive genetic disorder caused by mutations in the gene encoding the  $\alpha$ -chain polypeptide of the lysosomal enzyme B-hexosaminidase A. One in thirty Ashkenazi Jews are carriers for a severe form of the disease. Dogma assumed that all members of this ethnic group harbored the same mutation, but a splice junction defect and insertion defect in two different Ashkenazi patients, which could be detected in approximately 20% and 70% of the carriers, respectively, was identified. Moreover, the patient used to characterize the insertion defect was heterozygous for it and null for the splice junction defect, indicating the presence of a third mutation in the Ashkenazi population; the base change of G to A in exon 11 was shown to introduce a premature termination codon. The defect was not found among one hundred Ashkenazi carriers screened, suggesting that this is a very low frequency allele or a private family mutation.

I-cell disease is a recessive genetic disorder caused by a deficiency of N-acetylglucosaminylphosphotransferase, a membrane bound enzyme involved in equipping lysosomal enzymes with phosphomannosyl residues that serve to route these enzymes to lysosomes. Since the phosphotransferase has been shown to recognize lysosomal enzymes as specific substrates by means of a protein determinant shared by these enzymes, attempts will be made to affinity purify the phosphotransferase via its binding to the lysosomal enzyme  $\beta$  hexosaminidase B, which will be linked to a gel matrix. To obtain sufficient quantities of  $\beta$ -hexosaminidase-B for this purpose, active enzyme was produced in cultured insect (Sf9) cells by isolation of a recombinant insect virus (Baculovirus) containing the  $\beta$ -chain cDNA, and infection of Sf9 cells with this construct. The  $\beta$ -hexosaminidase B was purified and characterized with regards to size, N-terminal sequence, in vivo processing and oligosaccharide structure. It was shown that the recombinant enzyme can serve as a substrate for the phosphotransferase thus it will be used to purify that enzyme.



The REV protein of HIV has been recently shown to lead to premature transport of the partially spliced mRNA encoding the envelope glycoprotein from the nucleus to the cytoplasm. To develop a system to examine the effects of REV, a means has been devised for generating nuclei in vitro around exogenously added DNA. This method involves using extracts from Xenopus laevis where it has been demonstrated that nuclear assembly can be reconstituted. The availability of such preparations should allow examination of the mechanism of REV function. In other studies, examination has begun of the structure of the nuclear pore complex, across which REV, TAT, and other viral proteins cross following viral infection. The structure of the pore complex is explored using recombinant DNA techniques. The major nuclear pore glycoprotein np62 has been cloned, sequenced and expressed in cultured cells. The gene encoding this protein has been isolated and found to be 2.95 kb in length and devoid of introns. The availability of the primary sequence of this protein has allowed the preparation of anti-peptide antibodies which react with p62. These antibodies should prove useful for probing the function of the pore complex in the HIV life cycle.

### The Role of the Nuclear Envelope in Intracellular Protein Sorting

Macromolecular transport across the nuclear membrane plays a central role in the regulation of eukaryotic cell growth and development. The mechanism of nuclear transport is under investigation. Recently, nuclear localization sequences have been identified which selectively target proteins to the nucleus. An in vitro binding and import assay has been developed using isolated rat liver nuclei and peptide conjugates labelled with colloidal gold or with <sup>125</sup>I. Conjugates having the proper sequence bind to nuclei with a K<sub>d</sub>=100nM. The nuclear membrane is a self assembling structure; when DNA is added to extracts of eggs of Xenopus laevis, nuclei form which resemble those of normal cells. The labelled conjugates enter these reformed nuclei. This system is being exploited to analyze the requirements for nuclear transport. The nuclear pore complex mediates transport across the nuclear membrane. The laboratory has demonstrated that proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. To examine the function of these glycoproteins in nuclear transport, the cDNA encoding the major pore protein p62 was cloned. The gene encoding p62 is devoid of introns and has two potential transcription start sites. p62 has two domains: one myosin-like and one collagen-like. By transfecting an expression construct encoding p62 this protein has been overexpressed in cultured cells. Using the cloned cDNA it has also been demonstrated that O-linked GlcNAc addition can be catalyzed in vitro in a rabbit reticulocyte lysate.

Efforts are underway to identify the yeast homologue of p62 so that yeast genetics can be used to determine the normal function of p62. This will be combined with studies involving the addition of recombinant rat p62 to the Xenopus nuclear reconstitution assay.

### Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

Thyroid hormone synthesis depends on a complex series of steps. These include transport of iodide, iodination and storage of thyroglobulin, and the processing of thyroglobulin with release of thyroid hormones. Earlier studies in this laboratory had concentrated on the first step in thyroid hormone synthesis by characterizing iodide transport and the regulation of this

process by TSH and adrenergic agents. Studies over the last several years have concentrated on the events subsequent to this transport. One study involves familial goiter in an exotic animal, bongo antelope. Several of these animals housed at the National Zoo have a thyroglobulin with an abnormal protein structure that is responsible for their goiters. Another study involves endemic goiter due to the absence of dietary iodine. Despite differences in etiology of these two goiters, i.e. abnormal thyroglobulin and iodine deficiency, both were found to secrete large amounts of albumin and cytochrome into the follicular lumen. Regulation of this abnormal secretion, and its relation to normal thyroid hormone synthesis is the direction of our present work. Also studied was thyroid tissue from a patient with congenital goiter. This patient's thyroglobulin is apparently normal in structure as well as in its acceptor function for iodination. Hypothyroidism in this patient is, however, associated with asialo-thyroglobulin. This observation is the first suggesting that sialic acid may be critical for normal processing of thyroglobulin leading to release of thyroid hormones.

#### Cell Regulation by Pharmacodynamic and Autoimmune Agents Acting on Cell Membranes

This laboratory is studying the structure-function relationships of (a) the thyrotropin (TSH) receptor, (b) receptors for other glycoprotein hormones and (c) receptors on the thyroid which also regulate thyroid function and growth and function, i.e. receptors for alpha 1-adrenergic agents, cholinergic agents, and insulin or insulin-like growth factors. The aim is to identify (a) receptor determinants important for ligand interactions, receptor cross-talk, and signal transduction, (b) transcriptional and posttranscriptional mechanisms by which TSH and other ligands affect gene expression and cell function, and (c) receptor determinants important for the expression and development of thyroid autoimmune disease, its complications, i.e. exophthalmos or pretibial myxedema, organ-specific autoimmunity (Lupus, diabetes), and thyroid tumors. The rat and human TSH receptors have now been cloned. Epitopes on the receptor recognized by autoantibodies in immune thyroid disease have been distinguished; regulation of receptor gene expression has been defined; and the role of the signal peptide in processing but not TSH binding, resolved. A heat shock 70 protein as well as gamma-actin have been recognized to be related to TSH receptor structure-function; and a new Graves' nuclear autoantigen has been characterized which binds TSH and DNA, whose mRNA and antigenic expression correlates with thyroid cell growth, goiter, and oncogene tumorigenicity in the thyroid, and which is identical to the Ku autoantigen of lupus. Related studies have resolved the role of different signal transduction mechanisms in thyroid cell growth; the transport thyroid hormones from the lysosome; and the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis. For example, the induction of the cyclooxygenase and prostaglandin pathway has been shown to require TSH, insulin/IGF-I and serum, and to correlated with the time course and hormonal requirements of thyroid cell growth.  $\text{PGE}_2$  is both a critical signal for DNA synthesis, a negative feedback regulator of cAMP mediated thyroid function, and an altered modulator in "aging" cells.

### III. REGULATION OF DNA EXPRESSION BY PROTEINS

The interaction of specific proteins with DNA is probably the major regulatory guide of growth and differentiation. These effects have been demonstrated for a specific physiological function, lactation, and for viral behaviour.

#### Tissue Specific and Hormone Regulated Gene Expression

1. The molecular basis of mammary-specific and hormone regulated gene expression is being studied through analyses of regulatory elements of the mouse whey acidic protein (WAP) gene using transgenic animals. There is evidence to suggest that prolactin induces milk protein gene expression during pregnancy by a post-transcriptional mechanism. To test this, a mouse WAP gene allele, different from the endogenous one, was introduced into transgenic mice, and its expression was analyzed during pregnancy and upon prolactin stimulation. Whereas the endogenous WAP gene was strongly induced in the second half of pregnancy and by prolactin, expression of the transgene was considerably less affected. This suggests that induction of WAP gene expression during pregnancy and upon hormonal stimulation is mainly regulated at the transcriptional level and that the corresponding element(s) is located several kb from the promoter.

In continuation of establishing the mammary gland as a bioreactor the human CD4 receptor was expressed in milk of transgenic mice. A model system for transgenic farm animals was established with altered milk protein composition. Transgenic pigs carrying the mouse WAP gene were generated and high levels of WAP were detected in milk, suggesting that it is possible to express foreign proteins in milk of transgenic dairy animals.

2. The enhancer of the immediate early 1 gene of the human cytomegalovirus contains several repeated and unique transcription elements. Since it is possible that transcription elements may interact with each other to fully activate the gene, synergism between such elements and between different signalling pathways was investigated. Specifically, NF1 and kB elements, can synergistically activate transcription in several cell types. In addition, the cAMP response element was synergistically activated by cAMP and PHA, suggesting convergence of the protein kinase A and C pathways on this transcription element.

#### Cell Specific Activity of Elements within the HIV-LTR

The suggestion that HIV may be activated upon exposure to steroid hormones is the clinical observation that HIV p24 antigenemia is more frequently found in pregnant rather than non-pregnant women and that this antigenemia disappears after parturition. Transcription from the HIV-LTR was therefore studied in the presence or absence of hormonal stimuli in tissue culture cells and transgenic animals. In the presence of dexamethasone, transcription from the HIV-LTR in tissue culture cells was elevated at least 4-fold. Moreover expression from the HIV-LTR was elevated in transgenic elements during pregnancy (1) suggesting that steroid hormones may contribute to HIV expression during pregnancy.

#### IV. PHYSICAL ASPECTS OF MEMBRANES, TRANSPORT AND PROTEINS

The more physical attributes of proteins, lipids, and DNA, as well as their interaction in membranes and function in transport, is the concern of several groups in the Laboratory.

##### Physics of Ionic Channels and other Proteins with Aqueous Cavities

Membrane channel proteins appear to "open" and "close" by collapsing the aqueous cavity through which ions flow. That picture, derived from earlier studies on mitochondrial and axon proteins, has now been seen by osmotic stress studies with the alpha-toxin protein. Widening the purview of the investigation on proteins whose control derives from different accessibility of the surface to water solvent, a shift in the uptake of oxygen by hemoglobin has been measured when the molecule is subject to lowered activity of water. In this way, an increase of 60 to 80 water molecules were found to be associated with hemoglobin when it loads oxygen.

An apparatus was developed to detect membrane electrical "noise" as a source of information on the activity of large populations of channels.

##### Direct Measurement of Forces between Membranes or Macromolecules

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular assembly and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, DNA double helices, and stiff linear polysaccharides.

Until now, only force or energy vs. surface separation between molecules or membranes has been measured. During the current year, the "entropy" and "enthalpy" of release water, as a function of the distance between macromolecules, has been successfully extracted.

The first phase of a systematic study of forces between polysaccharides has also been completed. Again, for these materials as for nucleic acids and lipids, exponentially decaying "hydration" forces are seen between molecules approaching contact.

A theory of lipid bilayer forces that claims to relate the "dipole potential" set up by the lipid polar groups to the perturbation of water near the lipid interface is being tested. A combined osmotic stress, x-ray diffraction, NMR and ion transport study shows that the correlation demanded by the theory does not exist.

Bilayers of synthetic lipids, thought from the work of others not to show strong hydration forces, have now shown a very strong version of this force when observed by our direct, osmotic stress method.

Finally, a surprisingly easy transition between lamellar and non-lamellar packing of lipids, whose occurrence may be related to easy rearrangement of cell membrane lipids in secretion and fusion, is being investigated.

## Cell-Cell Fusion Due to Influenza Hemagglutinin

Infection of animal cells by enveloped viri is accomplished in part when the viral genome is delivered into the cell following the fusion of the cell and viral membranes. This membrane fusion event is catalysed by a protein: in the case of influenza the hemagglutinin (HA). In addition to viral-cell membrane fusion, HA will also mediate the fusion of cells, provided the molecule exists in sufficient concentration in at least one of the two cell membranes, is activated by appropriate limited proteolysis, and is protonated at  $\text{pH} < 5$ . Fusion between cells expressing influenza virus hemagglutinin (HA protein), and human red blood cell (RBC) was detected by the transfer and dequenching of both a lipid marker and a cytoplasmic marker. Both measures of fusion, lipid continuity and cytoplasmic continuity, were evident only after a delay: about 30 sec at 37 deg and  $\text{pH} 4.9$ . The studies outlined above have been continued by following single cell-cell fusion reactions in various ways. Examining fusion at the level of single events adds information which was not appreciated hitherto: 1) Fusion is all or nothing: either a pair will fuse at a given  $\text{pH}$ , or after a pulse, or it will not; there is no evidence of partial fusion. 2) Reacidification from neutrality, following failure to fuse, leads to fusion with the same lag time and time-course; lag time is independent of history of exposure to  $\text{pH}$ . 3) Individual fusion lag times and time-courses are broadly heterogeneous, both in rate and shape of curve. 4) Fusion is initiated with a small, reversible pore. These facts provide new constraints on the possible mechanisms of fusion. Primarily, the fact that a small increase in proton concentration caused a large increase in the percentage of fusing cells, but does not change the lag time implies that protons are playing the role of an allosteric modulator. The fact that the reaction continues after a short pulse of protons indicates the fusion, at some point in the reaction cascade, is irreversible. The fact that pore formation is reversible and variable during pore widening makes the viral protein induced membrane fusion similar to the membrane fusion pore seen in exocytosis.

## Histamine Release and Hydration of Granule Matrices

Mast cells of beige mice present with intracellular secretory granules of approximately 4 microns in diameter. When individual granules fuse with the plasma membrane during exocytosis, the cell surface area increases by 2-8 % for each fusion event (as measured by monitoring plasma membrane capacitance) and the granule diameters increase by 40%. These cells have been used in two independent but related projects.

In the first exocytotic pores were studied which form an aqueous pathway between the granule interior and the extracellular medium. The frequency histogram for occurrence of pores of given conductance is broad and independent of the frequency of the stimulating sine wave used to measure capacitance. Furthermore the frequency histogram is similar for two simultaneous but independent measurements of capacitance.

In the second project, the physico-chemical nature of granule swelling was studied. Secretory granules generally contain a dehydrated, condensed polymer network or matrix that hydrates and swells upon release. In beige mouse mast cells, relative hydration is dependent upon the constituents of its ionic environment. Divalent cations reduce hydration whereas monovalent cations

either have a negligible effect or increase the polymer hydration relative to the effects of distilled water alone. The size is critically dependent upon the solution present at the moment of initial swelling. This variable state behavior may have clinical relevance for cystic fibrosis, a genetic disorder in which abnormally thick, dehydrated mucus secretions obstruct airways and pancreatic ducts. If the hydration state of the mucus is also irreversibly dependent on initial extracellular ionic conditions at the time of mucus secretion, then patients may have mucus in an altered state due to the well known alteration in ionic composition of the extracellular composition.

#### Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanism

This laboratory is engaged in studies on protein structure and function. Specific questions include how a protein chain, which is synthesized as a random coil can fold to a highly specific secondary and tertiary structure without aid from any external agents. The blueprint for these higher order structures must be encoded in the proteins primary sequence. This mechanism of protein folding is being investigated by studies on the behaviour of swine pepsinogen, studying the kinetics of its folding and unfolding reactions and the structure of partially folded intermediates observed in these reactions. Enzymic activity in folded proteins can be modulated or even totally suppressed by covalent modifications to the proteins structure. Two examples under investigation include activation of pepsinogen to the active enzyme pepsin by cleavage of the polypeptide chain, and modulation of the various activities of myosin by phosphorylation of both its heavy and light chains by a number of kinases, which are themselves under metabolic control. The sites of these reactions and their consequences are being studied.

#### Structure and Physical Properties of DNA and DNA-Protein Complexes

The physical structure of an activated gene complexes is as yet unknown, although the formation of DNA loops or bends stabilized by protein binding or protein - protein interactions is considered likely. Previously the binding of divalent Zn to the 5s RNA gene from *Xenopus laevis* was shown to induce a strong bend in the DNA. The magnitude and center position of this bend from a series of DNA fragments with circularly permuted sequences and rotational relaxation times measured by transient electric birefringence have now been determined. The bend is some 50-60 degrees and is centered at about base pair +60 within the gene. This places the bend within the binding domain of the transcription regulatory factor TFIIIA. The rotational relaxation time of the TFIIIA-5s RNA gene complex is only slightly faster than the decay time found for the Zn-DNA complex. The binding of the regulatory factor is most probably stabilizing the same bent DNA conformation as Zn binding. Solution conditions that inhibit the formation of the Zn induced bend in DNA also inhibit the binding of TFIIIA to the gene. This is the first example of a bent DNA - protein complex that is stabilized by the formation of an alternate DNA conformation. The DNA sequence that appears responsible for the bent conformation is GGG. This triplet is prominent in several regulatory DNA binding sequences, most notably the spl binding domain of SV40. Further work is underway both to characterize the sequence requirements for the bent conformation, using a series of single base mutations in the TFIIIA binding domain of the 5s RNA gene, and the generality of inducing a bent conformation at this sequence by the binding of transcription regulatory proteins, using the 21 bp sequence from SV40.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17001-24 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of the Carbohydrate Moiety of Glycoproteins in Cellular Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Ashwell

Institute Scholar LBM, NIDDK

## COOPERATING UNITS (if any)

Free University, Berlin, West Germany

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The hepatic receptor for asialoglycoproteins was found to be modulated by the glucose concentration in the medium of the human hepatoma cell line, HepG2. The surface binding of asialoorosomucoid increased from 20 ng/mg of cellular protein to about 40 ng/mg as the glucose concentration was increased from 10 to 50 mg %. Scatchard plot analysis indicated a rise in the number of binding sites as well as a two-fold increase in binding affinity. The binding of antibody against the human receptor did not change, confirming that the actual number of receptors remained constant in face of an increased number of binding sites. Specificity of the glucose effect was indicated by the binding of insulin and transferrin to their respective receptors, which was unaffected under the conditions that increased asialoglycoprotein binding. The up-modulating effect of glucose was abolished by 2-deoxyglucose, an inhibitor of glucose metabolism, and by cycloheximide, an inhibitor of protein synthesis. The mechanism responsible for the modulation is currently unknown, although presumptive evidence is available to suggest that it is mediated by changes in the level of cyclic GMP.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17002-20 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic Basis of Detoxication

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: W.B. Jakoby Chief, LBM LBM, NIDDK

Others: Y-S. Yang Visiting Fellow LBM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Among the thirty or so enzymes of detoxication is the group of esterases which are ubiquitously distributed among cells, with about 95 per cent of total activity present in the microsomal fraction of liver. The esterases fall into two classes based on catalytic mechanism. The major activity by far is with the serine-type esterases whereas a minor fraction has a different, as yet unknown, hydrolytic mechanism.

An investigation of those enzyme that are in rat liver cytosol has resulted in the isolation of two electrophoretically homogeneous proteins that have a high affinity for aspirin and probably account for most of the hydrolysis of that ester in liver. These are serine-esterases which also catalyze the hydrolysis of a large variety of other esters.

A systematic examination of the second group, non-serine esterases, in the cytosol allowed the isolation of twelve esterases of varying molecular size and isoelectric point, all relatively uninhibited by paraoxon, but able to accommodate a very wide range of substrates with an overlapping specificity. Four of these enzymes were prepared in homogenous form and antibody was obtained against three of them; each antibody reacted only with the esterase that served as antigen and not with any of the other eleven esterases. In addition to the oxygen esters, thiol esters served as substrates but amides and peptides did not.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17003-23 LBM

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Polysaccharides in Morphogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Cabib	Senior Research Chemist	LBM, NIDDK
Others:	E. Mol	Visiting Fellow	LBM, NIDDK
	J.T. Mullins	Special Volunteer	LBM, NIDDK
	H.-M. Park	Visiting Fellow	LBM, NIDDK
	J.A. Shaw	IRTA	LBM, NIDDK
	S.J. Silverman	Expert	LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Developmental Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL

4.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two chitin synthetases, Chs1 and Chs2, are known to be present in yeast. Chs2 is essential for septum formation and cell division. The previous hypothesis, that Chs1 has a repair function during cell separation, has been now confirmed by unambiguous genetic evidence.

The genes coding for the two chitin synthetases, CHS1 and CHS2, share an extensive homologous region but differ in the amino terminal sequence. By gene deletions and ligations we have determined that the amino terminal region of CHS2 is not required for either enzymatic activity or function.

Genetic analysis has confirmed that proteinase B is not required for activation of the Chs1 zymogen in vivo. Chs1 has been found to be more sensitive than Chs2 to certain antifungal agents.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17004-22 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Peter McPhie Research Chemist LBM, NIDDK

COOPERATING UNITS (if any) LMC, NHLI (Robert Adelstein); LCP, NIDDK (Edith Miles); MB, NCI (Jane Cheng); Fidia Institute, Georgetown University (Alla Berkowich); Dept of Biochemistry, Georgetown University (Preston Hensley, Assoc. Prof.); LPD, NIAID (Russell Howard); LPS, DCRT (Richard Shrager)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This laboratory is engaged in studies on protein structure and function. Specific questions include how a protein chain, which is synthesized as a random coil can fold to a highly specific secondary and tertiary structure without aid from any external agents. The blueprint for these higher order structures must be encoded in the proteins primary sequence. This mechanism of protein folding is being investigated by studies on the behaviour of swine pepsinogen, studying the kinetics of its folding and unfolding reactions and the structure of partially folded intermediates observed in these reactions. Enzymic activity in folded proteins can be modulated or even totally suppressed by covalent modifications to the proteins structure. Two examples under investigation include activation of pepsinogen to the active enzyme pepsin, by cleavage of the polypeptide chain and modulation of the various activities of myosin by phosphorylation of both its heavy and light chains by a number of kinases, which are themselves under metabolic control. The sites of these reactions and their consequences are being studied.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17008-07 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of the Nuclear Envelope in Intracellular Protein Sorting

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J.A. Hanover	Research Chemist	LBM, NIDDK
Others:	M. D'Onofrio	Visiting Fellow	LBM, NIDDK
	C. Starr	Guest Researcher	LBM, NIDDK
	M. Miller	IRTA	LBM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

3.2

## PROFESSIONAL

3.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Macromolecular transport across the nuclear membrane plays a central role in the regulation of eukaryotic cell growth and development. The mechanism of nuclear transport is under investigation. Recently, nuclear localization sequences have been identified which selectively target proteins to the nucleus. An *in vitro* binding and import assay has been developed using isolated rat liver nuclei and peptide conjugates labelled with colloidal gold or with  $^{125}\text{I}$ . Conjugates having the proper sequence bind to nuclei with a  $K_d=100\text{nM}$ . The nuclear membrane is a self assembling structure; when DNA is added to extracts of eggs of *Xenopus laevis*, nuclei form which resemble those of normal cells. The labelled conjugates enter these reformed nuclei. This system is being exploited to analyze the requirements for nuclear transport. The nuclear pore complex mediates transport across the nuclear membrane. The laboratory has demonstrated that proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. To examine the function of these glycoproteins in nuclear transport, the cDNA encoding the major pore protein p62 was cloned. The gene encoding p62 is devoid of introns and has two potential transcription start sites. p62 has two domains: one myosin-like and one collagen-like. By transfecting an expression construct encoding p62 this protein has been overexpressed in cultured cells. Using the cloned cDNA it has also been demonstrated that O-linked GlcNAc addition can be catalyzed *in vitro* in a rabbit reticulocyte lysate.

Efforts are underway to identify the yeast homologue of p62 so that yeast genetics can be used to determine the normal function of p62. This will be combined with studies involving the addition of recombinant rat p62 to the *Xenopus* nuclear reconstitution assay.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17009-05 LBM

PERIOD COVERED  
October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Tissue Specific and Hormone Regulated Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)  
PI: L. Hennighausen Visiting Associate LBM, NIDDK

Others: T.G. Burdon Special Volunteer LBM, NIDDK  
R.A. McKnight IRTA LBM, NIDDK  
H.H. Niller Visiting Fellow LBM, NIDDK  
A. Shamay Visiting Fellow LBM, NIDDK

COOPERATING UNITS (if any)  
US Department of Agriculture (Robert Wall); Land O'Lakes (Leonard Ruiz); American Red Cross (William Drohan), FRG, Akademie der Wissenschaften (M. Schwerin)

LAB/BRANCH  
Laboratory of Biochemistry and Metabolism

SECTION  
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS 5	PROFESSIONAL 5	OTHER:
----------------------	-------------------	--------

CHECK APPROPRIATE BOX(ES)  
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

1. The molecular basis of mammary-specific and hormone regulated gene expression is being studied through analyses of regulatory elements of the mouse whey acidic protein (WAP) gene using transgenic animals. There is evidence to suggest that prolactin induces milk protein gene expression during pregnancy by a post-transcriptional mechanism. To test this, a mouse WAP gene allele, different from the endogenous one was introduced into transgenic mice and its expression was analyzed during pregnancy and upon prolactin stimulation. Whereas the endogenous WAP gene was strongly induced in the second half of pregnancy and by prolactin, expression of the transgene was considerably less affected. This suggests that induction of WAP gene expression during pregnancy and upon hormonal stimulation is mainly regulated at the transcriptional level and that the corresponding element(s) is located several kb from the promoter.

In continuation of establishing the mammary gland as a bioreactor the human CD4 receptor was expressed in milk of transgenic mice. A model system for transgenic farm animals was established with altered milk protein composition. Transgenic pigs carrying the mouse WAP gene were generated and high levels of WAP were detected in milk, suggesting that it is possible to express foreign proteins in milk of transgenic dairy animals.

2. The enhancer of the immediate early 1 gene of the human cytomegalovirus contains several repeated and unique transcription elements. Since it is possible that transcription elements may interact with each other to fully activate the gene, synergism between such elements and between different signalling pathways was investigated. Specifically, NF1 and kB elements, can synergistically activate transcription in several cell types. In addition, the cAMP response element was synergistically activated by cAMP and PHA, suggesting convergence of the protein kinase A and C pathways on this transcription element.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17024-07 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## The Genetic Lesions of Tay-Sachs Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Myerowitz Research Chemist LBM, NIDDK

Others: J.-A. Boose Staff Fellow LBM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Tay-Sachs disease is a recessive genetic disorder caused by mutations in the gene encoding the  $\alpha$ -chain polypeptide of the lysosomal enzyme B-hexosaminidase A. One in thirty Ashkenazi Jews are carriers for a severe form of the disease. Dogma assumed that members of this ethnic group harbored the same mutation, but a splice junction defect and insertion defect in two different Ashkenazi patients which could be detected in approximately 20% and 70% of the carriers, respectively was identified. Moreover, the patient used to characterize the insertion defect was heterozygous for it and null for the splice junction defect indicating the presence of a third mutation in the Ashkenazi population. A G to A base change in exon 11 which introduces a premature termination codon was identified. The defect was not found among one hundred Ashkenazi carriers screened. This suggests that the lesion is a very low frequency allele or private family mutation.

I-cell disease is a recessive genetic disorder caused by a deficiency of N-acetylglucosaminylphosphotransferase, a membrane bound enzyme involved in equipping lysosomal enzymes with phosphomannosyl residues that serve to route these enzymes to lysosomes. Since the phosphotransferase has been shown to recognize lysosomal enzymes as specific substrates via a protein determinant shared by these enzymes, attempts will be made to affinity purify the phosphotransferase via its binding to the lysosomal enzyme  $\beta$  hexosaminidase B, which will be linked to a gel matrix. To obtain sufficient quantities of  $\beta$ -hexosaminidase-B for this purpose, active enzyme was produced in cultured insect (Sf9) cells by isolation of a recombinant insect virus (Baculovirus) containing the  $\beta$ -chain cDNA, and infection of Sf9 cells with this construct. The  $\beta$ -hexosaminidase B was purified and characterized with regards to size, N-terminal sequence, in vivo processing and oligosaccharide structure. It was shown that the recombinant enzyme can serve as a substrate for the phosphotransferase thus it will be used to purify that enzyme.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18007-11 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.F. Grollman Medical Officer (Research) LBM, NIDDK

Others: S. Doi Visiting Fellow LBM, NIDDK  
M.C. Baggio Visiting Fellow LBM, NIDDK

## COOPERATING UNITS (if any)

LBM, NIDDK (L.D. Kohn); DBB, OBR (N.Y. Nguyen), USUHS (D. Tombaccini);  
Smithsonian Institute (R.J. Montali); Univ. Sao Paulo (Medeiros-Neto); Biomedical  
Institute, Madrid (P. Santisteban); EMBO, Heidelberg (R. DiLauro).

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Cell Regulation

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.25

## PROFESSIONAL

2.00

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Thyroid hormone synthesis depends on a complex series of steps. These include transport of iodide, iodination and storage of thyroglobulin, and the processing of thyroglobulin with release of thyroid hormones. Earlier studies in this laboratory concentrated on the first step in thyroid hormone synthesis by characterizing iodide transport and the regulation of this process by TSH and adrenergic agents. Studies over the last several years have concentrated on the events subsequent to this transport. One study involves familial goiter in an exotic animal, bongo antelope. Several of these animals housed at the National Zoo have a thyroglobulin with an abnormal protein structure that is responsible for their goiters. Another study involves endemic goiter due to the absence of dietary iodine. Despite differences in etiology of these two goiters, i.e. abnormal thyroglobulin and iodine deficiency, both were found to secrete large amounts of albumin and cytochrome into the follicular lumen. Regulation of this abnormal secretion, and its relation to normal thyroid hormone synthesis is the direction of our present work. Also studied was thyroid tissue from a patient with congenital goiter. This patient's thyroglobulin is apparently normal in structure as well as in its acceptor function for iodination. Hypothyroidism in this patient is, however, associated with asialo-thyroglobulin. This observation is the first suggesting that sialic acid may be critical for normal processing of thyroglobulin with release of thyroid hormones.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18008-24 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Regulation by Pharmacodynamic and Autoimmune Agents Acting on Cell Membranes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	L.D. Kohn	Medical Director, USPHS, and Chief, Sec on Cell Regulation	LBM, NIDDK
Others:	T. Akamizu	Visiting Fellow	LBM, NIDDK
	S. Ikuyama	Visiting Fellow	LBM, NIDDK
	S. Kosugi	Visiting Fellow	LBM, NIDDK
	M. Saji	Visiting Fellow	LBM, NIDDK
	K. Tahara	Visiting Fellow	LBM, NIDDK

COOPERATING UNITS (if any) NIDDK (E.F. Grollman, V. Nikodem); U. Pisa, Italy (A. Pinchera); U. Naples (R. DiLauro, E. Avvedimento, S. Aloj, & E. Consiglio); NIAID (C. Kozak); NCI (W. McBride); CCHD (W. Gahl); NIDR (B. Prabhakar); U. MD (W.A. Valente); U. of Kyoto, Japan (T. Mori).

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Cell Regulation

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.3

## PROFESSIONAL:

5.3

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

This laboratory is studying the structure-function relationships of (a) the thyrotropin (TSH) receptor, (b) receptors for other glycoprotein hormones and (c) receptors on the thyroid which also regulate thyroid function and growth and function, i.e. receptors for alpha 1-adrenergic agents, cholinergic agents, and insulin or insulin-like growth factors. The aim is to identify (a) receptor determinants important for ligand interactions, receptor cross-talk, and signal transduction, (b) transcriptional and posttranscriptional mechanisms by which TSH and these other ligands affect gene expression and cell function, and (c) receptor determinants important for the expression and development of thyroid autoimmune disease, its complications, i.e. exophthalmos or pretibial myxedema, organ-specific autoimmunity (Lupus, diabetes), and thyroid tumors. This year, the rat and human TSH receptors have been cloned. Epitopes on the receptor recognized by autoantibodies in immune thyroid disease have been distinguished; regulation of receptor gene expression has been defined; and the role of the signal peptide in processing but not TSH binding, resolved. A heat shock 70 protein as well as gamma-actin have been recognized to be related to TSH receptor structure-function; and a new Graves' nuclear autoantigen has been characterized which binds TSH and DNA, whose mRNA and antigenic expression correlates with thyroid cell growth, goiter, as well as oncogene tumorigenicity in the thyroid, and which is identical to the Ku autoantigen of lupus. Related studies have resolved the role of different signal transduction mechanisms in thyroid cell growth; the transport thyroid hormones from the lysosome; and the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis. For example, the induction of the cyclooxygenase and prostaglandin pathway has been shown to require TSH, insulin/IGF-I and serum and correlated with the time course and hormonal requirements of thyroid cell growth. PGE<sub>2</sub> is both a critical signal for DNA synthesis, a negative feedback regulator of cAMP mediated thyroid function, and an altered modulator in "aging" cells.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18009-11 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. R. Robbins	Research Geneticist	LBM, NIDDK
Others:	C.W. Hall	Research Chemist	LBM, NIDDK
	C.F. Roff	Senior Staff Fellow	LBM, NIDDK
	T.M. Weber	Staff Fellow	LBM, NIDDK

## COOPERATING UNITS (if any)

LI, NIDR, NIH (Dr. Constance Oliver)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL

3.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An approach to dissecting the processes of endocytosis, glycoprotein biosynthesis and sorting is through isolation and analysis of mutants. Based on observations of aberrant compartmentalization of the M6P/IGFII receptor in a temperature-sensitive End1 mutant, perturbation of the intracellular pathways followed by this receptor was attempted. Summarizing these results: 1. In the presence of weak base human diploid fibroblasts accumulate up to 40% of receptor-enzyme complexes in dense lysosomes; this accumulation represents a steady state and is rapidly reversed upon re-acidification of organelles. 2. Inhibition of protein synthesis effects rerouting of lysosomal enzymes present in the secretory pathway from a predominantly intracellular to a predominantly endocytic route of delivery to lysosomes. 3. Inhibition of protein synthesis also blocks accumulation of receptor-ligand complexes in lysosomes and effects a futile cycle whereby these complexes shuttle between the cell surface and an intracellular compartment(s). It is hypothesized that a rapidly turning-over protein is required for delivery of receptor-enzyme complexes to the late endosome via both the endocytic and intracellular pathways.

Electron microscopic examination of endocytosed tracers in a CHO cell mutant isolated for its accumulation of ligand in non-acidic vacuoles indicates that the mutant may be impaired in vesicular fusion.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18010-03 LBM

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Intracellular Traffic in HIV Infection

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: J.A. Hanover Research Chemist LBM, NIDDK

Others: M. Miller IRTA LBM, NIDDK

COOPERATING UNITS (# any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL 1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

CD4, the T-cell receptor for the human immunodeficiency virus, depends upon glycosylation for proper surface expression. Initial studies employing acute lymphoblastic leukemic cells were extended by the successful transfection of a plasmid containing the cDNA for CD4 into mutant and wild type Chinese hamster ovary cells. Characterization of these clones suggests that CD4 contains biantennary unsialylated complex-type oligosaccharides. The data also suggest that the inability of CD4 to reach the cell surface when it lacks carbohydrate is due to the selective degradation of unglycosylated CD4.

The REV protein of HIV has been recently shown to lead to the premature transport of the partially spliced mRNA encoding the envelope glycoprotein from the nucleus to the cytoplasm. To develop a system to examine the effects of REV, a means has been devised for generating nuclei *in vitro* around exogenously added DNA. This method involves using extracts from *Xenopus laevis* where it has been demonstrated that nuclear assembly can be reconstituted. The availability of such preparations should allow examination of the mechanism of REV function. In other studies, examination has begun of the structure of the nuclear pore complex, across which REV, TAT, and other viral proteins cross following viral infection. The structure of the pore complex is explored using recombinant DNA techniques. The major nuclear pore glycoprotein np62 has been cloned, sequenced and expressed in cultured cells. The gene encoding this protein has been isolated and found to be 2.95 kb in length and devoid of introns. The availability of the primary sequence of this protein has allowed the preparation of anti-peptide antibodies which react with p62. These antibodies should prove useful for probing the function of the pore complex in the HIV life cycle.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18011-03 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Specific Activity of Elements within the HIV-LTR

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: L. Hennighausen Visiting Associate LBM, NIDDK

## COOPERATING UNITS (if any)

NICHD (Heiner Westphal); NCI (Priscilla Furth)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

One clinical suggestion that HIV may be activated upon exposure to steroid hormones is the observation that HIV p24 antigenemia is more frequently found in pregnant rather than non-pregnant women and that this antigenemia disappears after parturition. Transcription from the HIV-LTR was therefore studied in the presence or absence of hormonal stimuli in tissue culture cells and transgenic animals. In the presence of Dexamethasone transcription from the HIV-LTR in tissue culture cells was elevated at least 4-fold. Moreover expression from the HIV-LTR was elevated in transgenic elements during pregnancy (1) suggesting that steroid hormones may contribute to HIV expression during pregnancy.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18012-06 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Direct Measurement of Forces between Membranes or Macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PIs:	V.A. Parsegian	Guest Researcher	LBM, NIDDK
	D.C. Rau	Expert	LBM, NIDDK

Others:	K. Gawrisch	Visiting Fellow	DCRT
	S. Leikin	Visiting Fellow	DCRT
	D. Ruston	Special Volunteer	LBM, NIDDK
	J.J. Zimmerberg	Sr. Research Investigator	LBM, NIDDK

## COOPERATING UNITS (if any)

Brock Univ., Ontario (R.P. Rand); Univ. British Columbia, Vancouver (E.A. Evans); Univ. Minnesota (D.F. Evans); Princeton University (S.M. Gruner); Tulane University (W. Reed); Naval Research Laboratory (W. O'Grady)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

3.4

## PROFESSIONAL

3.4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular assembly and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, DNA double helices, and stiff linear polysaccharides.

Until now, only force or energy vs. surface separation between molecules or membranes has been measured. During the current year, the "entropy" and "enthalpy" of release water, as a function of the distance between macromolecules, has been successfully extracted.

The first phase of a systematic study of forces between polysaccharides has also been completed. Again, for these materials as for nucleic acids and lipids, one sees exponentially decaying "hydration" forces between molecules approaching contact.

A theory of lipid bilayer forces that claims to relate the "dipole potential" set up by the lipid polar groups to the perturbation of water near the lipid interface is being tested. A combined osmotic stress, x-ray diffraction, NMR and ion transport study shows that the correlation demanded by the theory does not exist.

Bilayers of synthetic lipids, thought from the work of others not to show strong hydration forces, have now shown a very strong version of this force when observed by our direct, osmotic stress method.

Finally, a surprisingly easy transition between lamellar and non-lamellar packing of lipids whose occurrence may be related to easy rearrangement of cell membrane lipids in secretion and fusion is being investigated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18013-03 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physics of Ionic Channels and other Proteins with Aqueous Cavities

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: V.A. Parsegian Guest Researcher LBM, NIDDK

Others: S. Bezrukov Special Volunteer LBM, NIDDK  
M.F. Colombo Visiting Fellow LBM, NIDDK  
J.J. Kasianowicz IRTA LBM, NIDDK  
C.R. Moore IRTA LBM, NIDDK  
D.C. Rau Expert LBM, NIDDK  
J.J. Zimmerberg Sr. Research Investigator LBM, NIDDK

## COOPERATING UNITS (if any)

Rockefeller University (Sanford M. Simon, Ph.D., Gunther Blobel, Ph.D.); Hopkins University (Andrew Harris, Ph.D.); Office of Naval Research (Igor Vodyanoy, Ph.D.); Y. Steinberg (Fogarty Scholar); St. George's Hospital, University of London (Charles Pasternak, Lindsay Bashford)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

5.5

## PROFESSIONAL

5.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Membrane channel proteins appear to "open" and "close" by collapsing the aqueous cavity through which ions flow. That picture, derived from earlier studies on mitochondrial and axon proteins, has now been seen via osmotic stress studies on the alpha-toxin protein.

Widening the purview of our investigation on proteins whose control derives from different accessibility of the surface to water solvent, we have measured a shift in the uptake of oxygen by hemoglobin when the molecule is subject to lowered activity of water. In this way we have measured an increase of 60 to 80 water molecules associated with hemoglobin when it loads oxygen.

During the past year, too, we have developed apparatus to detect membrane electrical "noise" as a source of information on the activity of large populations of channels.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18014-06 LBM

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Physical Properties of DNA and DNA-Protein Complexes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: D.C. Rau Expert LBM, NIDDK

## COOPERATING UNITS (if any)

LCB, NHLBI (Dr. E. Korn); George Mason University, Fairfax, VA (Dr. H. Chen); LMB, NIDDK (Dr. J. Nickol); LCP, NIDDK (Drs. M. Riehm and E. Charney); University of Nevada, Reno, Nevada (Dr. R. Harrington); Univ. of Calgary, Alberta, Canada (Dr. D. Bazett-Jones)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

.67

## PROFESSIONAL

.67

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The physical structure of an activated gene complexes is as yet unknown, although the formation of DNA loops or bends stabilized by protein binding or protein - protein interactions is considered likely. Previously the binding of divalent Zn to the 5s RNA gene from *Xenopus laevis* was shown to induce a strong bend in the DNA. The magnitude and center position of this bend from a series of DNA fragments with circularly permuted sequences and rotational relaxation times measured by transient electric birefringence have now been determined. The bend is some 50-60 degrees and is centered at about base pair +60 within the gene. This places the bend within the binding domain of the transcription regulatory factor TFIID. The rotational relaxation time of the TFIID-5s RNA gene complex is only slightly faster than the decay time found for the Zn-DNA complex. The binding of the regulatory factor is most probably stabilizing the same bent DNA conformation as Zn binding. Solution conditions that inhibit the formation of the Zn induced bend in DNA also inhibit the binding of TFIID to the gene. This is the first example of a bent DNA - protein complex that is stabilized by the formation of an alternate DNA conformation. The DNA sequence that appears responsible for the bent conformation is GGG. This triplet is prominent in several regulatory DNA binding sequences, most notably the spl binding domain of SV40. Further work is underway both to characterize the sequence requirements for the bent conformation, using a series of single base mutations in the TFIID binding domain of the 5s RNA gene, and the generality of inducing a bent conformation at this sequence by the binding of transcription regulatory proteins, using the 21 bp sequence from SV40.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18015-06 LBM

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histamine Release and Hydration of Granule Matrices

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: M.J. Curran Staff Fellow LBM, NIDDK

Others: J. Zimmerberg Senior Research Investigator LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.75

PROFESSIONAL

1.75

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mast cells of beige mice present with intracellular secretory granules of approximately 4 microns in diameter. When individual granules fuse with the plasma membrane during exocytosis, the cell surface area increases by 2-8 % for each fusion event (as measured by monitoring plasma membrane capacitance) and the granule diameters increase by 40%. These cells have been used in two independent but related projects.

In the first project, these exocytotic pores were studied which form an aqueous pathway between the granule interior and the extracellular medium. The frequency histogram for occurrence of pores of given conductance is broad and independent of the frequency of the stimulating sine wave used to measure capacitance. Furthermore the frequency histogram is similar for two simultaneous but independent measurements of capacitance.

In the second project, the physico-chemical nature of granule swelling was studied. Secretory granules generally contain a dehydrated, condensed polymer network or matrix that hydrates and swells upon release. In beige mouse mast cells, relative hydration is dependent upon the constituents of its ionic environment. Divalent cations reduce hydration whereas monovalent cations either have a negligible effect or increase the polymer hydration relative to the effects of distilled water alone. This size is critically dependent upon the solution present at the moment of initial swelling. This variable state behavior may have clinical relevance for Cystic Fibrosis (CF), a genetic disorder in which abnormally thick, dehydrated mucus secretions obstruct airways and pancreatic ducts. If the hydration state of the mucus is also irreversibly dependent on initial extracellular ionic conditions at the time of mucus secretion, then patients with CF may have mucus in an altered state due to the well known alteration in ionic composition of the extracellular composition.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18016-03 LBM

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell-Cell Fusion Due to Influenza Hemagglutinin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: J. Zimmerberg Senior Research Investigator LBM, NIDDK

Others: M.J. Curran Staff Fellow LBM, NIDDK

COOPERATING UNITS (if any)

NCI/LTB (R. Blumenthal); Foreign: Tel Aviv University (D. Kaplan)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL

1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Infection of animal cells by enveloped virus is accomplished in part when the viral genome is delivered into the cell following the fusion of the cell and viral membranes. This membrane fusion event is catalysed by a protein: in the case of influenza the hemagglutinin (HA). In addition to viral-cell membrane fusion, HA will also mediate the fusion of cells, provided the molecule exists in sufficient concentration in at least one of the two cell membranes, is activated by appropriate limited proteolysis, and is protonated at  $\text{pH} < 5$ . Fusion between cells expressing influenza virus hemagglutinin (HA protein), and human red blood cell (RBC) was detected by the transfer and dequenching of both a lipid marker and a cytoplasmic marker. Both measures of fusion, lipid continuity and cytoplasmic continuity, were evident only after a delay: about 30 sec at 37 deg and  $\text{pH} 4.9$ . The studies outlined above have been continued by following single cell-cell fusion reactions in various ways. Examining fusion at the level of single events adds information which was not appreciated hitherto: 1) Fusion is all or nothing: either a pair will fuse at a given  $\text{pH}$ , or after a pulse, or it will not; there is no evidence of partial fusion. 2) Reacidification from neutrality, following failure to fuse, leads to fusion with the same lag time and time-course; Lag time is independent of history of exposure to  $\text{pH}$ . 3) Individual fusion lag times and time-courses are broadly heterogeneous, both in rate and shape of curve. 4) Fusion is initiated with a small, reversible pore. These facts provide new constraints on the possible mechanisms of fusion. Primarily, the fact that a small increase in proton concentration caused a large increase in the percentage of fusing cells, but does not change the lag time implies that protons are playing the role of an allosteric modulator. The fact that the reaction continues after a short pulse of protons indicates the fusion, at some point in the reaction cascade, is irreversible. The fact that pore formation is reversible and variable during pore widening makes the viral protein induced membrane fusion similar to the membrane fusion pore seen in exocytosis.

## ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

### NATIONAL INSTITUTE OF DIABETES, DIGESTIVE AND KIDNEY DISEASES

#### SECTION ON BIOCHEMICAL MECHANISMS

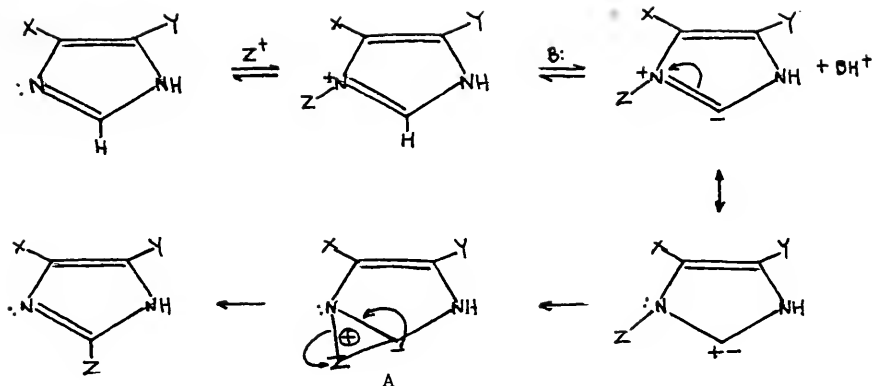
#### CHEMISTRY OF IMIDAZOLES AND BIOIMIDAZOLES

Over the past 15 years, we have found consistently that 2-X-bioimidazoles (especially X = fluoro and iodo) have a broad range of strong biological activities but the corresponding 4-X-bioimidazoles are essentially inactive. A number of 4-X-bioimidazoles are accessible by direct electrophilic substitution (nitro, halo, etc.); 2-X-bioimidazoles are far less accessible and can be obtained by indirect and, often, very tortuous routes. Prior to our major efforts in this area, the majority of 2-X-bioimidazoles were still unknown. By far the 2-substituted bioimidazoles most easily obtainable are the 2-iodo and 2-trifluoromethyl compounds. The 2-iodo derivatives of histidine and histamine are of interest, not only because of their potent antimalarial activities, but because they can serve as valuable intermediates for the synthesis of other 2-X-bioimidazoles, as can the trifluoromethyl analogs. We are currently exploring the utility of such conversions to prepare photosensitive bioimidazoles and affinity labels for in vivo use. Such studies could have been performed many years ago, but for the fact that these analogs had not been available through classical or obvious synthetic routes. Even methods suitable for simple imidazoles are not applicable to complex bioimidazoles, because of the additional functional groups and the desire to preserve chirality. Thus, nonclassical methods (e.g., photochemical radical substitution, electrochemistry, B-12 catalysis, one-electron reduction, etc.) were developed to fit these gaps.

Even more novel methods are always being sought to provide analogs still inaccessible, or to require conditions which even a polypeptide or polynucleotide can withstand. We have now developed procedures for the conversion of amino groups into azido and nitro or halo, of trifluoromethyl into methyl, cyano, carboxy, carbomethoxy, carbamido, etc. Recently, we synthesized 2- and 4-pentafluoroethylhistidine and histamine by photochemical radical substitution. These compounds are converted by base into the corresponding trifluoroacetyl derivatives, which have such reactive carbonyl groups that they may serve as affinity labels for bioimidazole-binding sites. Upon treatment with methanolic base, the trifluoromethyl analogs can be converted into trimethoxymethyl and the pentafluoroethyl derivatives into ketals. These ortho functionalities are also of interest as potential covalent affinity labels.

For many years, imidazole specialists have tried to explain why bromination and iodination at C-2 occur with great ease while chlorination is almost impossible. Our demonstration that C-2 does not undergo acid-catalyzed isotope exchange leads to the requirement that halogenation at C-2 cannot occur via the normal electrophilic mechanism. We have proposed, and are gathering evidence for, an alternative ylid mechanism (Scheme I). This mechanism would very easily explain why chlorination differs from other halogenations, since it is well known that the cyclic chloronium ion (A) is much more difficult to form than the other halonium ions.





SCHEME I

## ANTIMALARIALS

Our development, in 1971, of a photochemical route to ring-fluorinated aromatics and heteroaromatics has led to the synthesis of a wide variety of fluoro analogues of imidazole-based metabolites. Many of these compounds have shown interesting properties as agonists or antagonists and have proved useful as research tools and as possible chemotherapeutic agents. A striking difference was found between 2-fluoro-L-histidine (2-FHIS) and the 4-fluoro isomer. While the former compound is readily incorporated into new protein in place of histidine (both in bacteria and mammals), the 4-fluoro isomer is not incorporated at all. Further, 2-FHIS shows antibacterial, antiviral, antileukemic and antimalarial properties; again, 4-FHIS shows none of these activities. From our 13-C NMR studies of other substituted histidines, we now believe this biological differentiation to be based on tautomer preference in the imidazole ring; thus, while certain 2-X-histidines resemble histidine in preferring the 1,4-tautomer, 4-X and 2,4-di-X-histidines prefer the unnatural 1,5-tautomer. We have found such differentiation in a number of isomer pairs, including X = fluoro, iodo, trifluoromethyl, etc. Furthermore, 2-fluorohistamine was found to be a potent agonist at the histamine H-1 receptor, while 4-fluorohistamine is active at the H-2 receptor. Again, tautomer preference may be the basis for such differentiation and may provide a rationale for the design of other agonists and antagonists.

We have become particularly interested in the antimalarial properties of 2-FHIS, since the compound is uniquely and selectively active against Plasmodium falciparum, that parasite which is notoriously resistant to chemotherapy. The organism has the unusual property of inducing production, within an invaded erythrocyte, of a protein containing as much as 70% histidine. The protein is found in knobs which are seen on the erythrocyte surface; these knobs may be responsible for a very strong adherence of the infected erythrocyte to capillary endothelium, thereby sequestering parasitized cells which would normally be destroyed during passage through the spleen. In cultures of infected erythrocytes, low concentrations of 2-FHIS not only inhibit cytoadherence but prevent maturation of the parasite and the appearance of knobs entirely. The assumption that these antiparasitic properties are due to the incorporation of 2-FHIS into the histidine-rich protein is probably unwarranted, since the treated parasite shows a general decrease in protein synthesis and rather low incorporation of

labeled 2-FHIS. As one of several hypotheses for the mechanism of action, we propose that 2-FHIS interferes with histidine as a promoter of the transport of some other amino acids into the cell. This hypothesis is supported by our earlier findings that 2-FHIS inhibits protein synthesis in cell and organ cultures but not in cell-free systems. Studies are in progress on the effect of 2-FHIS on facilitated amino acid transport. Unfortunately, the high antimalarial activity shown by 2-FHIS in vitro could not be extended, because the compound proved too toxic to monkeys. Such toxicity is surprising, since mice tolerate as much as 500 mg/kg. We are now exploring derivatives of 2-FHIS in the hope of reducing toxicity.

A large number of other substituted histidines have been screened for in vitro antimalarial activity: 2-iodo showed good activity while 2-azido gave moderate activity. Once again, 4-X-histidines show no activity. Surprisingly, the 2-chloro and 2-bromo analogues are inactive. The role of the iodine atom may be steric or lipophilic (and probably not electronis). On the other hand, out 13-C NMR studies suggest that 2-bromoimidazoles may have an opposite tautomer preference from that of 2-iodo, a result which challenges explanation. Thus, the specificity of the 2-iodo derivative may be due to a remarkable tautomer preference. In order to attempt any structure-activity correlation, data is needed for other substituted histidines with large lipophilic, and metabolically stable, groups at C-2 (iPr, tBu, Ph, Bz, etc.). We are now developing new synthetic methods to obtain such compounds, based on the cyclization of 1,2-diacylaminoethylenes with acid chlorides and phase-transfer catalysis by dimethylaminopyridine.

#### SIGNIFICANCE OF LIGAND TAUTOMERISM IN BIORECOGNITION

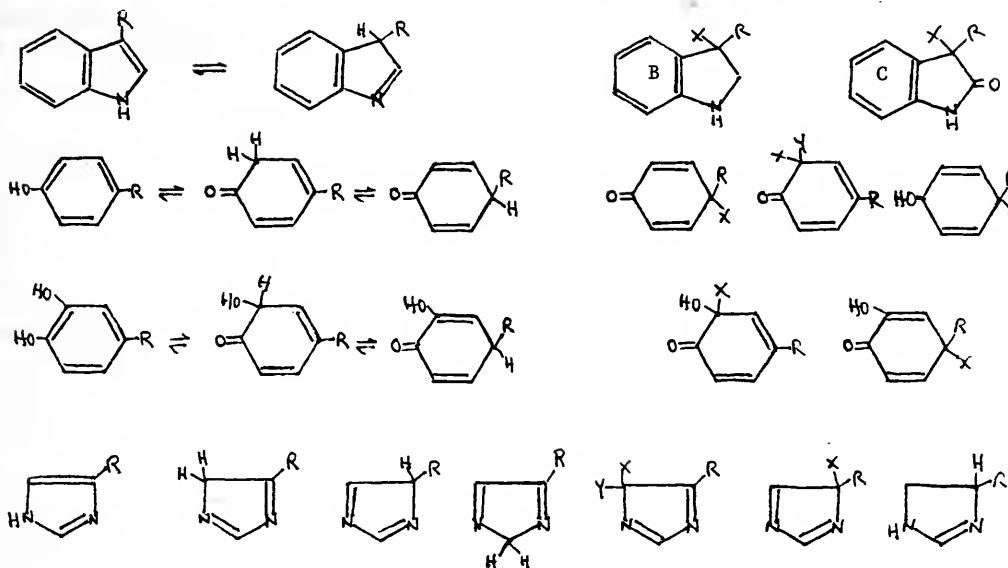
Many biologically important molecules exist as tautomer pairs, in which the major tautomer (tantotautomer) is so favored energetically over the minor tautomer (tenutautomer) that the latter species cannot even be detected in the equilibrium mixture by any spectroscopic means. Nevertheless, mechanistic studies have revealed that the tenutautomer is very often the "true" reactant in various chemical transformations. We have proposed that the same phenomenon may exist in living systems, namely, that the tenutautomers (Scheme II) of various metabolites may be the "true" ligands for some enzymes and receptors. This theory provides a new pathway for substrate activation; it also leads to the critical consequence that potential inhibitors and antagonists should be designed as analogues of the tenutautomers, rather than the common practice of mimicking the tantotautomers. Since it is still impossible to examine the detailed structure of a substrate within a binding site, arguments for the tenutautomer concept must be based on evidence and inference from the behavior of stable analogues of the tenutautomer.

This approach was highly successful and very convincing in the case of tryptophan. Stable analogues of the indolenine tautomer of tryptophan with a tetrahedral carbon at C-3—2,3-dihydro-L-tryptophan (B), oxindolyl-L-alanine (C, X = H) and dioxindolyl-L-alanine (C, X = OH)—are potent competitive inhibitors of tryptophan synthase and tryptophanase, enzymes involved in the biosynthesis and degradation of tryptophan. Furthermore, the two enzymes show "mirror-image specificity, in that the 3R diastereoisomer of the analogues inhibits only tryptophanase while the 3S diastereoisomer inhibits only tryptophan synthase. The diastereoisomers of 3-azido-oxindolyl-L-alanine have also been prepared as potential photoaffinity labels for these enzymes. A logical extension of this work involves the synthesis of tetrahedral analogues of the 5-hydroxy and 5-methoxy derivatives of biotryptophan, currently in progress.

## Tantotautomer

## Tenutautomers

## Some Tenutautomer Analogues



SCHEME II

Some years ago, we discovered a route to the p-hydroxydienone analogue of tyrosine by electrolytic oxidation (D, X = OH). Unfortunately, this compound is rather unstable and we are now exploring routes to more stable systems (D, X = OMe, CN, N<sub>3</sub>, OAc, alkyl, etc.). These analogues will be tested as inhibitors of tyrosine phenol-lyase. Even more important tests and applications of the principle involve the synthesis and evaluation of dienone analogues of catecholamines and dihydroxyphenylalanine; synthesis of such analogues presents a novel and unprecedented challenge.

## STEREOPOPULATION CONTROL IN DRUG DELIVERY AND ENZYME SIMULATION

Enzymes catalyze reactions  $10^{10}$  to  $10^{20}$  as fast as their uncatalyzed test-tube counterparts. In order to account for this remarkable catalytic power of enzymes, it is usually considered that the activation free energy needed to achieve the reaction is contributed both by binding of the substrate to the enzyme (step 1) and by chemical transformation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2. We have proposed, however, that the overall conversion process is more easily justified on the assumption that the first step contributes a more significant share of the activation energy. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions at rates approaching those promoted by enzymes. Most importantly, these test-tube reactions do not depend on any catalysis but succeed simply because the substrate is frozen in a highly favorable conformation. Such results demonstrate that an enzyme, by binding and freezing the substrate in a

single, rigid conformation, can raise both the entropic and enthalpic components of the free energy to the point of achieving transformation without requiring any catalytic involvement of protein functional groups.

Recent work has involved a study of steric and electronic effects on NMR and IR spectra across tight space rather than through covalent bonds. These studies show that spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limit of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo group. After considerable effort, this goal has been reached; a large variety of restricted systems have now been prepared and spectral/kinetic studies are in progress. The availability of these rigid, sterically crowded systems also provides a tool for the study of steric effects in the three-dimensional structure of a protein, by isolating a single interaction from the thousands of others. As part of our studies of practical applications of stereopopulation control, we have been exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport of drugs from the gut to the circulatory system to the brain, by temporary masking of charge within the molecule and by improvement in lipophilicity.

#### ALDOSE REDUCTASE INHIBITORS

Inhibition of the enzyme, aldose reductase, represents a recent pharmacological approach toward the treatment of late-onset diabetic complications. These complications affect the eye (cataracts), kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of high intracellular concentrations of sorbitol, in turn resulting from the reduction of excess glucose symptomatic of diabetes. Aldose reductase is the enzyme responsible for sorbitol formation.

Our studies on the synthesis of inhibitors of tryptophan-metabolizing enzymes have involved some oxindole derivatives which are somewhat analogous in overall geometry to certain commercial compounds now in advanced clinical trials as aldose reductase inhibitors. In vitro assays have revealed that these oxindoles, and some of their transformation products, are as potent as any others thus far designed. Although these chiral compounds are not amino acids or their derivatives, we have succeeded in the use of chymotrypsin to achieve their resolution by selective ester hydrolysis. The introduction of certain substituents on the benzene ring of the oxindole has been found to increase inhibitor activity several fold. Additional analogues have been prepared, which are expected to serve as affinity and photoaffinity labels for the enzyme. Current efforts are being devoted to improvements in synthesis yields, in resolution and in enhancement of lipophilicity, with the goal of achieving more effective penetration and transport to the sites of action in vivo. Since our inhibitor series is significantly different in structure and in functional groups from those which had reached clinical trials, we have strong hope that this series will not elicit the serious side effects which had caused many of the commercial candidates to be abandoned.

## ANALOGUES OF THYROTROPIN-RELEASING HORMONE

In addition to governing the release of thyrotropin and prolactin from the pituitary, TRH (L-pyroglutamyl-L-histidinyll-L-proline amide) is known to exert a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise in the treatment of circulatory shock and/or CNS ischemic damage, as a promoter of the regeneration of injured spinal cord, as an antidepressant and in the amelioration of symptoms associated with amyotrophic lateral sclerosis (ALS). However, the great variety of its biological effects presents a serious drawback to its use as a drug for specific purposes. Our early studies with synthetic analogues of TRH (involving modification of the imidazole ring of histidine) had suggested that some of the central actions of TRH are not mediated through high affinity TRH receptors and that appropriate analogues may achieve desired specificity of action.

Receptor binding studies with these analogues have made it clear that endocrine and various centrally mediated actions of TRH involve uniquely different mechanisms and that, after a decade of effort in various laboratories, separation of these activities has been achieved. Thus, 4(5)-NO<sub>2</sub>-Im-TRH is highly selective for CVS activity, and may be useful in the treatment of various forms of shock without any endocrine effects. Conversely, Nva<sup>2</sup>-TRH is a selective analeptic compound without any effects on the cardiovascular system; this analogue has served as a useful research tool for delineation of those binding sites in rat brain which may mediate the analeptic effects of TRH and its analogues. Computer-assisted structure-activity analysis of a large number of imidazole-substituted analogues has helped us to design more selective and potent analogues, as well as photoaffinity labels for TRH receptors. Recently, we have carried out receptor binding analysis of various TRH analogues with subtle backbone modifications (replacement of the peptide amide with a thioamide surrogate); these studies have allowed us to identify subtle differences in the high affinity TRH receptors in rat pituitary and brain. Further receptor analysis and pharmacological studies with these compounds are expected to provide more insight into the mechanisms of TRH actions.

## FLUORINATED ANALOGUES OF BIOACTIVE PEPTIDES

Fluorinated analogues of a wide range of structurally different classes of organic compounds have been prepared and many of these have proven to be important pharmacological and medicinal agents. The advantages of fluorine substitution arise, primarily, from the small van der Waals radius of fluorine (1.35 Å). As a result, fluorine bonded to an sp<sup>2</sup> carbon is used effectively as a C-H replacement. Furthermore, because of similar aliphatic C-F and C-O bond lengths (1.39 and 1.43 Å, respectively) and because of the weak hydrogen-bond accepting property of the former, fluorine bonded to an sp<sup>3</sup> carbon has been used frequently as an OH surrogate, particularly in carbohydrate research. Thus, because of steric and some electronic similarities, fluorinated compounds often mimic their nonfluorinated parents with respect to recognition by biological macromolecular systems such as enzymes, transport proteins and receptors. On the other hand, the high electronegativity of fluorine (4.0) can drastically alter electron density distribution in the molecule which, in turn, affects pK values of neighboring functional groups and molecular dipole moments. All these altered physicochemical properties of the molecule, as a result of fluorine substitution, can result in drastically modified biological properties

such as potency and receptor selectivity. Furthermore, recent advances in  $^{19}\text{F}$ -NMR and electron energy-loss spectroscopic techniques, and  $^{18}\text{F}$ -positron emission tomography ( $^{18}\text{F}$ -PET), have increased the significance and application of Fluorinated molecules as biological markers.

Although many methods have been developed for fast and efficient incorporation of  $^{18}\text{F}$  and  $^{19}\text{F}$  into various compounds of medicinal importance, such methods in general have not been practical for direct incorporation into peptides. We have now developed a method for fast, efficient introduction of F regio-specifically into the phenolic ring of Tyr-containing peptides by use of the electrophilic fluorinating agent, acetyl hypofluorite ( $\text{AcOF}$ ). We have used this method for fluorinating  $\mu$ -selective opioid peptides. In vitro bioassays and receptor binding assays have revealed that these fluoro peptides retain their high receptor selectivity. Thus, we have been able to develop a very  $\mu$ -selective fluoropeptide [ $\text{Tyr}(3\text{-F})\text{-D-Arg-Phe-Lys-NH}_2$ ], which may be a useful research tool to probe the complex opioid receptor system.

#### NOVEL AMINO ACIDS FOR CONFORMATIONAL AND STEREOCHEMICAL CONSTRAINTS IN PEPTIDES

Introduction of conformational restraints in peptides is a very useful approach to probe peptide-receptor interactions and to enhance their potencies and/or selectivity. While local conformational restraints can be imposed by incorporating dehydro- or cyclopropane amino acids, side chain-side chain cyclization to form cyclic analogues has been found to be one of the most useful approaches for introducing general conformational restraints. The use of a disulfide bond between Cys or Pen residues, or an amide bond between side chains of amino and carboxy trifunctional amino acids, are examples. The 13-membered cyclic peptide,  $\text{H-Tyr-D-Orn-Phe-Asp-NH}_2$ , is a potent  $\mu$ -selective opioid peptide. Molecular modeling of this peptide has revealed considerable flexibility in the ring structure, making it difficult to identify a single conformation which may be biologically relevant. In order to restrict further the conformational mobility in such peptides, we have designed a novel trifunctional amino acid,  $\beta$ -(2-pyrrolidinyl)alanine (PDA). This amino acid, if used in place of Orn or Lys for side chain-side chain cyclization, is expected to reduce considerably the conformational flexibility in the macrocyclic ring of these peptides owing to the introduction of a bicyclic structure. Moreover, the additional asymmetric center in the pyrrolidine ring may introduce differential stereochemical constraints to the binding of diastereoisomeric peptides, derived from the corresponding diastereoisomeric PDA's. Thus, these peptides may act as probes for delineating the stereochemical topology of the receptor in the vicinity of the ring structure and may have interesting biological properties.

Optically active amino acids are being used increasingly as synthons for preparation of a variety of chiral compounds. PDA's can be expected to be excellent synthons for compounds such as pyrrolizidines with the stereochemistry already defined at two centers. Furthermore, these amino acids may possess antimicrobial and/or antimetabolic activities. An efficient method for the synthesis of all four optically active stereoisomers of PDA has been developed. These amino acids will be used for the preparation of novel bicyclic opioid peptide analogues.

## SECTION ON CARBOHYDRATES

The Section continues its work on the interaction of (complex) carbohydrate determinants with receptors and monoclonal antibodies (MAbs). The elucidation of this interaction - in great molecular detail - is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. The approach uses:

1. The interaction of HIV gp120 with monocyte-receptors.
2. Physico-chemical studies on antibody/antigen systems.
3. The synthesis of ligands for affinity labeling.
4. The synthesis of ligands for detailed group interaction-studies.
5. The manipulation of immunoglobulin genes to produce specifically mutated genes expressing altered antibodies.
6. The study of immunodeterminants of bacteria causing significant diseases on a global scale, so as to evaluate procedures for vaccine development.

## RECENT WORK

### Sub 1.

The use of oligo-mannose containing neoglycoproteins prepared by us as competitive binders in the HIV gp120/CD4 receptor interaction has led to the proposal that the virus requires binary binding of both a protein- and a carbohydrate-determinant in order to infect (Kathleen Clouse's work). The work is continuing.

### Sub 2.

Deoxy- and deoxyfluoro derivatives (see also sub. 3) were used to map antidextran monoclonal (hybridoma) antibodies. Detailed binding contacts have been proposed for the antigenic determinant in the binding site of the the antibody. In the case of monoclonal antibodies IgA 16.4.12E and IgA W3129, this is the first time that antibodies that are specific for the the non-reducing terminal of a carbohydrate antigen, have been mapped in such detail by solution studies. This project continues by evaluation of an intercatenarily binding IgA (35.8.2H) with its antigen. Once that is finished, the dextran-anti-dextran work should come to a close (work by Eugenia Nashed, Eva Petráková, Pavol Kováč and Cornelis Glaudemans).

On the anti-galactan/galactan interactions: Measurements on a large number of synthetic ligands bearing deoxy-groups (see sub 3) has confirmed the binding patterns previously deduced from the deoxyfluoro derivatives. Thus, our novel method of frame-shifting ligands for the detection and mapping of ligand/antibody interactions, has been validated (work by Thomas Ziegler, Vilo Pavliak, Cornelis Glaudemans and Pavol Kováč).

### Sub 3.

The interaction of diazirino derivatives of galacto-oligosaccharides and anti-galactan immunoglobulins is continuing. The protein, after affinity-labeling, is fragmented and resulting peptides are separated for sequence analysis (work by Eugenia Nashed and Cornelis Glaudemans). New affinity labels of great complexity have been prepared, and were shown to bind well with the monoclonal antigalactans (work by Jochen Lehmann and Cornelis Glaudemans).

### Sub 4.

A large number of deoxy-derivatives of galactosyl (oligo)saccharides have been prepared. These derivatives, by virtue that they lack a hydroxyl group at defined positions disallow hydrogen bonding to *and* from that group with the protein. By the study of the binding of a large number of these, the method of selective frame-shifting to reveal protein-ligand interaction was fully confirmed (work by Pavol Kováč, Vilo Pavliak and Eva Petráková and Cornelis Glaudemans).

Sub 5.

After successfully cloning the  $v_H$  and  $v_L$  genes of antagalactan IgA X24, work is continuing on the expression of the oligonucleotide-mutated genes in the proper vector (work by Rao Arrepalli and Cornelis Glaudemans).

Sub 6.

*Shigella dysenteriae* Type 1 is an organism that is pathogenic in man only, and is the leading cause of enteric disease in the world. The Section has started an extensive program on mapping the interaction of the antigen with antibodies, with a view to assist in the development of a safe vaccine of excellent efficacy. We have affinity-purified antibody and obtained its Fab' fragment. We have studied its interaction with the entire antigen. In addition we are presently preparing all the combinations of oligosaccharidic determinants by a process of sophisticated synthetic procedures. Next we will prepare the appropriately derivatized ligands that will allow us to map the interaction of the immuno-determinant in the antibody's combining area (work by Pavol Kováč, Vilo Pavliak, Vincent Pozsgay and Cornelis Glaudemans).



## SECTION ON DRUG-RECEPTOR INTERACTIONS

### Biological Properties of Fluorinated Amines.

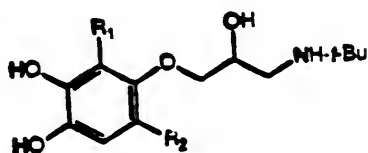
#### In Vivo Adrenergic Properties of Fluorinated Epinephrines.

We reported previously that fluorination of epinephrine (EPI) at the 6-position dramatically decreases agonist potency at  $\beta$ -adrenergic receptors and significantly increases potency at  $\alpha$ -adrenergic receptors, while fluorination at the 2-position markedly decreases potency at  $\alpha$ -adrenergic receptors and increases potency at  $\beta$ -adrenergic receptors. These fluorine-induced adrenergic selectivities had been demonstrated through specific isolated organ assays and through inhibition of binding of specific adrenergic ligands. We now have extended these results to include the effects of ring fluorination of EPI on the *in vivo* cardiovascular adrenergic receptor activities in the anesthetized dog. Increments in heart rate and contractile force were used as measures of  $\beta_1$ -adrenergic activity, while increases and decreases in blood pressure and decreases and increases in femoral blood flow were used to measure  $\alpha$ - and  $\beta_2$ -adrenergic activity, respectively. 2-F-EPI was three to five times more potent than EPI in stimulating an increase in heart rate ( $\beta_1$  response) while 6-FEPI actually elicited a slight decrease in heart rate. 2-F-EPI similarly caused a larger increase in cardiac contractile force than did EPI, while 6-EPI was much less effective. In contrast, 6-FEPI much greater increase in mean arterial pressure ( $\alpha$  response) than did EPI, while 2-FEPI showed a decrease in mean arterial pressure ( $\beta_2$ -response). EPI and 2-FEPI caused an increase in femoral blood flow ( $\beta_2$  response) while 6-FEPI caused a decrease in femoral blood flow ( $\alpha$  response). These data demonstrate clearly that fluorine substitution on EPI produces analogs that are specific and significantly more potent *in vivo* for adrenergic receptor subtypes. In particular, the selectivity and increased potency of 6-FEPI at  $\alpha$ -adrenergic receptors has made this analog very useful in several on-going pharmacological and electrophysiological studies.

#### Fluorinated Adrenergic Agonists as Probes for Mechanisms Drug-Receptor Interactions.

In every example of phenethanolamine adrenergic agonists (type A adrenergic agonists) we have examined, we have observed that fluorine at the 6-position greatly reduces binding of the agonist at  $\beta$ -adrenergic receptors, while fluorine in the 2-position has a similar effect at  $\alpha$ -adrenergic receptors. We also found that fluorine at the 6-position (**1c**) of 2-(1-butylamino)-1-(3,4-dihydroxyphenoxy)-2-propanol (**1a**) (a type B  $\beta$ -adrenergic agonist) causes a greatly reduced affinity at  $\beta$ -adrenergic receptors, while fluorine at the 2-position (**1b**) significantly increases affinity. This similar response to fluorine substitution has been used as evidence that fluorine-induced adrenergic selectivities are not caused by interactions of the fluorine with the side-chain, but more likely are caused by electronic alterations in the aromatic nucleuse. In addition, these results may have considerable significance with respect to the relative modes of binding of type A and type B  $\beta$ -adrenergic agonists. For example, the conformation shown in Figure 1 was proposed to explain the potent  $\beta$ -adrenergic agonist

properties of type B agonists.<sup>1</sup> In this conformation, the catechol ring and side chain functional groups can be superimposed on the corresponding sites of a phenethanolamine agonist in its favored extended conformation. Kaiser *et al.* noted that this formulation transposes the relative positions of aromatic substituents in the two series. Based on evidence from analog studies that the 3- and 4-positions of the aromatic ring occupy the same relative positions when bound to the receptor, they proposed the hydrogen bonded structure shown in Figure 2.<sup>2</sup> Additional investigations in several laboratories have failed to define precisely the structural correspondence between the two agonists types. However, our results suggest that the conformation shown in Figure 1 is unlikely, since the effects of fluorine substitution indicate that the 2- and 6-positions in each agonist type occupy the same position when bound to the receptor.



- a:  $R_1 = F, R_2 = H$   
 b:  $R_1 = H, R_2 = F$   
 c:  $R_1 = R_2 = H$

1a-c

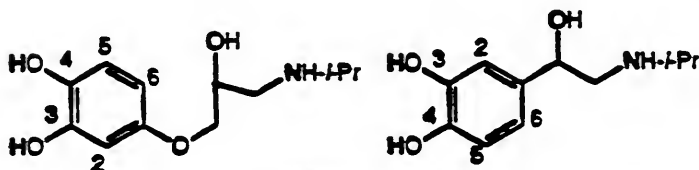


Figure 1

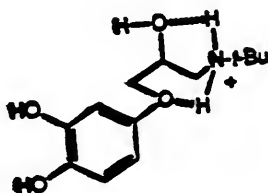
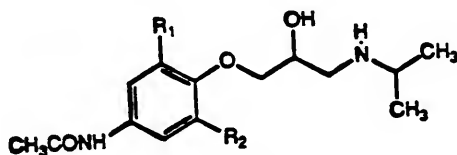


Figure 2

## Effects of Fluorine Substitution on Adrenergic Antagonists.

While certain structural features confer potent agonist properties to aryloxypropanolamines (N-alkyl substitution and the presence of the catechol ring), these compounds more often function as adrenergic antagonists. The demonstration that fluorine substitution effects the adrenergic agonist properties of phenoxypropanolamine adrenergic agonists prompted us to determine if fluorinated phenoxypropanolamine antagonists would show similar behavior. We prepared 2-fluoro- and 2,6-difluoro analogs (**2a,b**) of the potent  $\beta$ -adrenergic antagonist, practolol (**2c**) (Figure 4). Both fluorinated analogs had essentially the same affinity for  $\beta$ -adrenergic receptors as did the parent, indicating that the positions ortho to the ether linkage in this antagonist do not correspond to the same positions in the agonists **1a,b**. This would be consistent with the generally held view that agonists and antagonists have different binding modes at  $\beta$ -adrenergic receptors.

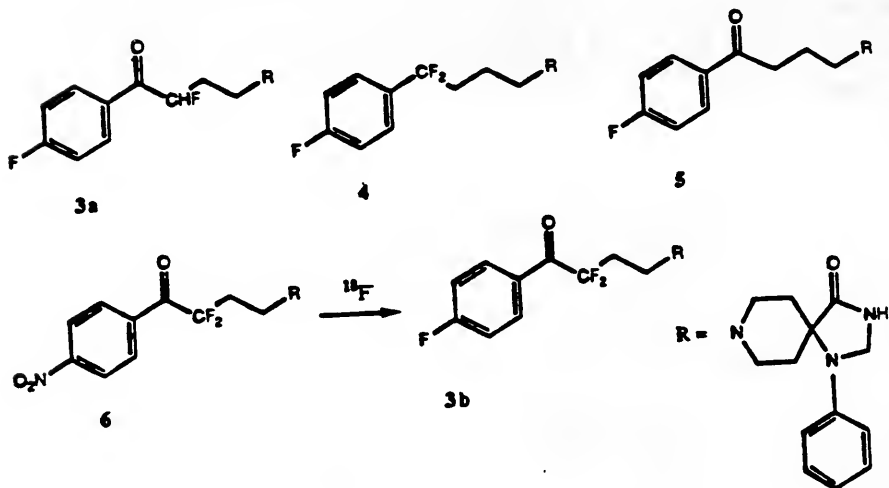


- 2a.**  $\text{R}_1 = \text{F}, \text{R}_2 = \text{H}$   
**b.**  $\text{R}_1 = \text{R}_2 = \text{F}$   
**c.**  $\text{R}_1 = \text{R}_2 = \text{H}$

## Fluorinated Spiperone.

Several considerations prompted us to prepare the side-chain fluorinated analogs **3a,b** and **4** of dopaminergic ligand spiperone (**5**). The *p*-fluorobutyrophenone moiety is critical to the binding of a series of neuroleptics, of which spiperone is an important member. The carbonyl carbon of  $\alpha,\alpha$ -difluoroketones has greatly increased electrophilicity relative to non-fluorinated ketones, a fact that has been exploited in the development of several important active site-directed enzyme inhibitors. If the carbonyl carbon of **5** is important to its binding to the dopamine receptor, increased electrophilicity in **3a** or **3b** might lead to an increased affinity. In addition, [ $^{18}\text{F}$ ]-labelled spiperone, and related ligands, have been used as PET-scanning agents for central dopamine receptors. Direct displacement of the nitro group in a precursor such as **6** with no carrier added [ $^{18}\text{F}$ ]-F has not been possible, likely because enolization of the carbonyl group under conditions of the displacement reaction blocks nucleophilic displacement of the *p*-nitro group. Since such enolization is not possible in **3b**, we envisioned using such a direct route to [ $^{18}\text{F}$ ]-labelled **3b**, provided **3b** retained

significant binding affinity. Analog 8 was synthesized to explore the possibility that the  $\text{CF}_2$  group could function as a mimic of the carbonyl group in this series. While all analogs possessed significant affinity for dopamine receptors, decreased affinities relative to spiperone place further applications in doubt.



### Fluorination of Biological Molecules.

We are continuing our research on direct fluorination of biological molecules using a variety of fluorinating agents. As part of this program, we have shown that acetyl hypofluorite can be used to introduce fluorine regiospecifically and efficiently ortho to OH in tyrosine-containing peptides. We have demonstrated this by the synthesis of  $\mu$ -selective opioid peptides derived from dermorphin, including Tyr(3-F)-D-Ala-Phe-Gly-NH<sub>2</sub> and Tyr(3-F)-D-Arg-Phe-Lys-NH<sub>2</sub>. The biological potencies of fluorinated analogs are reduced 2-7 fold relative to the parent peptides, but their selectivity of  $\mu$ -opioid receptors was essentially unchanged.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z 01 DK 19001-18LC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Reactions and Immunochemistry of Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Cornelis P. J. Glaudemans, Chief SOC

Others: R. S. Arepalli  
G. D. Daves  
P. Kováč  
E. M. Nashed  
V. Pavliak  
E. Petráková

Visiting Scientist  
Special Volunteer  
Research Chemist  
Visiting Scientist  
Visiting Fellow  
Visiting Associate

V. Pozsgay  
T. Ziegler

Visiting Scientist  
Special Volunteer

## COOPERATING UNITS (if any)

J. B. Robbins NICHD  
K. Clouse NIAID

J. Lehmann Freiburg University  
E. A. Kabat Columbia University

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Carbohydrates

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md 20892

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

5.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Section continues its work on the interaction of antigens with (monoclonal) antibodies. The elucidation of this interaction in great molecular detail is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. We are pursuing:

1. Physico-chemical studies.
2. The synthesis of ligands for affinity labeling.
3. The synthesis of ligands for detailed group interaction-studies..
4. The manipulation of immunoglobulin genes to produce specifically mutated genes expressing altered antibodies.
5. The study of immunodeterminants of bacteria causing significant diseases on a global scale so as to evaluate procedures for vaccine development.

The results of these studies are that the interaction of dextran antigens with their monoclonal antibodies (MAbs), both intercatenary- and end-binders, have been mapped in great molecular detail. The work on the interaction of antigalactan MAbs with their determinants has been finished, except for the preparation of mutated antibody genes, which continues. Also continuing is the work on affinity labeling of monoclonal antibodies. Extensive work is underway on the mapping of MAbs to *Shigella dysenteriae* Type 1 in order to generate an effective vaccine for this globally significant disease. Finally, work on the interaction of the gp120 of HIV with monocytes is continuing, and has led to a proposal of binary binding as a necessary prelude for infection.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 19603-14 LC
PERIOD COVERED October 1, 1989 to September 30, 1990		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) Analogues of Thyrotropin-releasing Hormone		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Virender M. Labroo	Special Expert NIDDK-LC
Others:	Louis A. Cohen	Chief, Section on Biochem. Mech. NIDDK-LC
	Stefan Vonnhof	Visiting Fellow NIDDK-LC
COOPERATING UNITS (if any) A. Spatola, Louisville, KY; C. Stammer, Athens, GA; A. Siren, USUHS; I. Paakari, Helsinki, Finland; A. Faden, San Francisco, CA; Y. Tache, Los Angeles, CA; G. Feuerstein, USUHS		
LAB/BRANCH Laboratory of Chemistry		
SECTION Section on Biochemical Mechanisms		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS 1.7	PROFESSIONAL 1.6	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>In addition to governing the release of thyrotropin and prolactin from the pituitary, TRH (L-pyroglyutamyl-L-histidinyl-L-proline amide) is known to exert a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise in the treatment of circulatory shock and/or CNS ischemic damage, as a promoter of the regeneration of injured spinal cord, as an antidepressant and in the amelioration of symptoms associated with amyotrophic lateral sclerosis (ALS). However, the great variety of its biological effects presents a serious drawback to its use as a specific drug. Our early studies with synthetic analogues of TRH (involving modification of the imidazole ring of histidine) have suggested that some of the central actions of TRH are not mediated through high affinity TRH receptors and that appropriate analogues may achieve desired specificity of action.</p> <p>Receptor binding studies with these analogues has made it clear that endocrine and various centrally mediated actions of TRH involve uniquely different mechanisms and that after a decade of effort in various laboratories, separation of these activities has been achieved. Thus, 4(5)-NO<sub>2</sub>-Im-TRH is highly selective for CVS activity and may be useful in the treatment of various forms of shock without any endocrine effects. Similarly, Nva<sup>2</sup>-TRH is a selective analeptic compound without any effects on cardiovascular system and has served as a useful research tool in delineating binding sites in rat brain which possibly mediate the analeptic effects of TRH and its analogues. Computer assisted structure-activity analysis of various imidazole-substituted analogues has helped us to design more selective and potent analogues as well as photoaffinity labels for TRH receptors.</p> <p>Recently we have carried out receptor binding analysis of various TRH analogues with subtle backbone modifications (replacement of peptide bond with thioamide surrogate) and have been able to identify subtle differences in the high affinity TRH receptors in rat pituitary and brain. Further receptor analysis and pharmacological studies with these compounds are expected to provide more insight into the mechanism of TRH actions.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19604-20 LC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stereopopulation Control in Drug Delivery and Enzyme Simulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen Chief, Sec. Biochem. Mech. NIDDK-LC

Other: Michael M. King Special Volunteer GWU

## COOPERATING UNITS (if any)

Yoshio Ueno, Nagoya, Japan; Wieslaw Antkowiak, Poznan, Poland; Yoshio Takeuchi, Toyama, Japan; Walter Dürckheimer, Frankfurt, FRG; Mariannina Impicciatore, Parma, Italy

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

0.1

## PROFESSIONAL

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to account for the remarkable catalytic power of enzymes, it is usually considered that the activation free energy is contributed both by binding of the substrate to the enzyme (step 1) and by chemical transformation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2. We have proposed, however, that the overall catalytic process is more easily justified on the assumption that the first step contributes a more significant share of the activation energy. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, and show that the protein raises both the entropic and enthalpic components of the substrate by binding it in a single, rigid conformation. Recent work has involved a study of steric and electronic effects on NMR and IR spectra across tight space rather than through covalent bonds. These studies show that spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limit of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo group. After considerable effort, this goal has been reached; a large variety of restricted systems have now been prepared and spectral/kinetic studies are in progress. As part of our studies of practical application of stereopopulation control, we have been exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport from the gut to the circulatory system to the brain by the temporary masking of charge within the molecule and by improvement in lipophilicity.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 19605-14 LC
PERIOD COVERED October 1, 1989 to September 30, 1990		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chemistry of Bioimidazoles		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Louis A. Cohen	Chief, Section on Biochem. Mech. NIDDK-LC
Others:	Bianca Avramovitch	Visiting Fellow (term 8/31/90) NIDDK-LC
	Virender Labroo	Special Expert NIDDK-LC
COOPERATING UNITS (if any) H. Kimoto, Nagoya, Japan; R. Henkin, Washington, DC; E. DeClercq, Louvain, Belgium; A. Shanz, Rehovot, Israel; W. Nagai, Nagoya, Japan; S. Avramovici, Jerusalem, Israel; J. Retez, Karlsruhe, FRG; A. Phillips, Univ. Park, PA; W. Dürckheimer, FRG		
LAB/BRANCH Laboratory of Chemistry		
SECTION Section on Biochemical Mechanisms		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.2	0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>A number of 4-X-bioimidazoles are accessible by direct electrophilic substitution (nitro, halo, etc.); 2-X-bioimidazoles are far less accessible and can be obtained only by indirect and, often, very tortuous routes. Prior to our major efforts in this area, the majority of 2-X-bioimidazoles were unknown. By far the 2-substituted imidazoles most easily obtainable are 2-iodo and 2-(trifluoromethyl). The 2-iodo derivatives of histidine and histamine are of interest, not only because of their potent antimalarial activities, but because they can serve as valuable intermediates for the synthesis of other 2-X-bioimidazoles, as can the trifluoromethyl analogues. We are currently exploring the utility of such conversions to prepare photosensitive bioimidazoles and affinity labels for in vivo use.</p> <p>Over the past 15 years, we have found consistently that 2-X-bioimidazoles (especially fluoro and iodo) have a broad range of strong biological activities but the corresponding 4-X-bioimidazoles are essentially inactive. Various explanations for this remarkable selectivity in biorecognition have been invalidated on experimental grounds. Our analyses of <sup>13</sup>C NMR spectra have now revealed that the differentiation may be based solely on tautomer preference. While 4-X-imidazoles exist preferentially as the unnatural <math>\pi</math>-tautomers, 2-X-bioimidazoles exist preferentially as the natural <math>\tau</math>-tautomers. This discovery generates broad implications in many areas of drug design, e.g., selective antihistamines, imidazole-based antimalarials and antimicrobials, CVS and CNS-selective TRH analogues, and analogues based on pyrrole and triazole regioisomerism. Thus, novel synthetic methods are being explored to make available even more 2-X-bioimidazoles.</p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19606-14 LC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Halogenated Biogenic Amines in Biochemistry and Pharmacology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kenneth L. Kirk Chief, Drug Receptor Interactions NIDDK-LC

Other: David Hebel Visiting Fellow NIDDK-LC

Kenneth A. Jacobson Research Chemist NIDDK-LC

Bang-Hua Chen Visiting Fellow (Arr. 5/90) NIDDK-LC

Yun-jing Nie Special Volunteer NIDDK-LC

Vital Shetty Special Volunteer NIDDK-LC

COOPERATING UNITS (if any) L.A. Cohen, V. Labroo (LC-NIDDK); J. Daly, C.R. Creveling, F. Gusovsky (LBC-NIDDK); M. Channing, D. Kieseewetter (CC, Dept. Nuclear Med.); D.S. Goldstein (HE-NHLBI), I.J. Kopin (DIR-NINCDS); C. Fraser (NIAAA); M. Linnoila (NIAAA); S. Reppert, S. Rivkees (Harvard Medical School); R.S. Phillips (Univ. of Georgia)

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Drug Receptor Interactions

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.2

## PROFESSIONAL:

3.5

## OTHER:

0.7

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Biogenic amines play key roles in neurotransmission, metabolism, and in control of various physiological processes. Using a variety of synthetic methodologies, including novel procedures developed by us, we have prepared a series of biogenic amines with fluorine substituted at various ring-positions. By virtue of its very small size and high electronegativity, fluorine is a very favorable replacement for hydrogen in these analogs. The biological properties and usefulness of these ring-fluorinated biogenic amines have proved to be extremely rewarding and continue to find applications in a multitude of studies, including research on the mechanisms of transport, storage, release, metabolism, and modes of action of these amines. Of particular significance was the discovery that 6-fluoronorepinephrine is a selective alpha-adrenergic agonist and 2-fluoronorepinephrine is a selective beta-adrenergic agonist. Mechanisms considered to explain these results include: 1) a direct effect of the C-F bond on agonist-receptor interaction or 2) an indirect effect of the C-F bond on the conformation of the ethanolamine side-chain. The results of testing of new analogs synthesized to probe these mechanisms indicate that electronic effects may be more important than conformational factors. Fluorinated analogs are useful biological tracers. For example, [ $^{18}\text{F}$ ]-labeled 6-fluorodopamine, the biological precursor to 6-fluoronorepinephrine, has been found to be an excellent scanning agent for peripheral noradrenergic innervation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19607-08 IC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Significance of Ligand Tautomerism in Biorecognition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LC
Other:	Rita Labroo	Special Volunteer	GWU
	Edith Miles	Senior Investigator	NIDDK-LBP
	Virender Labroo	Special Expert	NIDDK-LC

## COOPERATING UNITS (if any)

Robert Phillips, Athens, GA; H. Kimoto, Nagoya, Japan; W. Dürckheimer, Frankfurt, FRG; Shelly Avramovici, Jerusalem, Israel; J. Flippen-Anderson, NRL, Washington, DC

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Many biologically important molecules exist as tautomer pairs, in which the major tautomer (tantotautomer) is so favored energetically over the minor tautomer (tenu-tautomer) that the latter species cannot even be detected by any spectroscopic means in the equilibrium mixture. Nevertheless, mechanistic studies have revealed that the tenu-tautomer is very often the "true" reactant in various chemical transformations. We have proposed that the same phenomenon may exist in living systems, namely, that the tenu-tautomers of various metabolites may be the "true" ligands for some enzymes and receptors. This theory leads to the critical consequence that potential inhibitors and antagonists should be designed as analogues of the tenu-tautomers, not of the tantotautomers as is common practice.

Our first effort to support the theory has met with highly gratifying success. Stable analogues of the indolenine tenu-tautomer of tryptophan with a tetrahedral carbon at C-3, 2,3-dihydro-L-tryptophan, oxindolyl-L-alanine and dioxindolyl-L-alanine, are potent competitive inhibitors of tryptophan synthase and tryptophanase, enzymes involved in the biosynthesis and degradation of tryptophan. Furthermore, the two enzymes show "mirror-image" specificity, in that the 3R diastereoisomer of the analogues inhibits only tryptophanase while the 3S diastereoisomer inhibits only tryptophan synthase. The diastereoisomers of 3-azido-oxindolyl-L-alanine have been prepared as potential specific photoaffinity labels for these enzymes.

A similar approach is now being used with tyrosine phenol-lyase: methods have been developed to synthesize stable p-dienone analogues of tyrosine and these analogues will soon be evaluated with the enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 19608-07 LC

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Functionalized Congeners of Bioactive Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. Jacobson	Research Chemist	NIDDK-LC
Other: B. Bradbury	IRTA Fellow	NIDDK-LC
D. Boring	IRTA Fellow	NIDDK-LC
J. Zimmet	Special Volunteer	NIDDK-LC
U. Kammula	Special Volunteer	NIDDK-LC
Y. Karton	Visiting Scientist	NIDDK-LC

COOPERATING UNITS (if any) J. Daly, NIDDK-LBC; J. Baumgold (GW Univ.); B. Madras (Harvard University); K. Rice, NIDDK-LMC; A. Jacobson, NIDDK-LMC; G. Stiles (Duke University)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.1

PROFESSIONAL:

2.7

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent work in our laboratory and in others has demonstrated that certain drugs may be attached to well-defined "carrier" molecules and still retain the ability to bind to the receptor site and effect biological activity. This synthetic strategy for the attachment of drugs to carriers is termed the "functionalized congener" approach. The "carrier" molecule may be many times larger than the parent drug; indeed there is practically no maximum size limitation for a fully potent analog. Unlike the prodrug approach or the immobilization of drugs for slow release, the "functionalized congener" approach is designed to produce analogs for which no metabolic cleavage step is necessary for activation. Moreover, the attachment of the drug to a "carrier" such as a peptide may result in the enhanced affinity at an extracellular receptor site and an improvement in the pharmacological profile of the parent drug.

This strategy is being employed in the design of new agonists and antagonists at muscarinic acetylcholine receptors. These ligands are synthesized and characterized and then screened for potency and selectivity in binding assays in functional assays.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 19610-03 LC														
PERIOD COVERED October 1, 1989 to September 30, 1990																
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Prosthetic Groups for Radiolabeling of Functionalized Drugs and Peptides</b>																
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">K. Jacobson</td> <td style="width: 30%;">Research Chemist</td> <td style="width: 15%;">NIDDK-LC</td> </tr> <tr> <td rowspan="3">Other:</td> <td>K. Kirk</td> <td>Research Chemist</td> <td>NIDDK-LC</td> </tr> <tr> <td>D. Boring</td> <td>IRTA Fellow</td> <td>NIDDK-LC</td> </tr> <tr> <td>K. Taylor</td> <td>Special Volunteer</td> <td>NIDDK-LC</td> </tr> </table>			PI:	K. Jacobson	Research Chemist	NIDDK-LC	Other:	K. Kirk	Research Chemist	NIDDK-LC	D. Boring	IRTA Fellow	NIDDK-LC	K. Taylor	Special Volunteer	NIDDK-LC
PI:	K. Jacobson	Research Chemist	NIDDK-LC													
Other:	K. Kirk	Research Chemist	NIDDK-LC													
	D. Boring	IRTA Fellow	NIDDK-LC													
	K. Taylor	Special Volunteer	NIDDK-LC													
COOPERATING UNITS (if any) J. Roth (NIDDK); M. Lesniak (NIDDK); R. Eastman (NIDDK); M. Channing (NM-CC); D. Kiesewetter (NM-CC); J. Daly (NIDDK); Y. Shai (Weizmann Institute); G. Stiles (Duke University)																
LAB/BRANCH Laboratory of Chemistry																
SECTION Section on Drug Receptor Interactions																
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																
TOTAL MAN-YEARS: 0.7	PROFESSIONAL: 0.5	OTHER: 0.2														
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The use of radioisotopes to label organic compounds for use in diagnostic nuclear medicine is well documented in the literature. It has been found that certain radiolabeled compounds will localize in the brain, heart, or in other target organ or tissues to a sufficient level to allow for imaging thereof. There has been increasing interest in finding compounds which will more effectively cross the blood-brain barrier, thus facilitating more efficacious imaging of the brain.</p> <p>Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabeled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as <sup>18</sup>F, <sup>123</sup>I, and <sup>125</sup>I, to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor.</p> <p>Positron emission tomography (PET) has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of <sup>18</sup>F requires rapid and efficient reaction and purification; since the half-life is only 110 minutes.</p>																

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19611-03 LC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Drugs Acting at Adenosine Receptors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. Jacobson	Research Chemist	NIDDK-LC
Other:	X. Ji	Special Volunteer	NIDDK-LC
	D. Boring	IRTA Fellow	NIDDK-LC
	M. Maillard	Special Volunteer	NIDDK-LC

## COOPERATING UNITS (if any)

J. Daly (NIDDK); P. Churchill (Wayne State); G. Stiles (Duke University); P. Morgan (NIMH), L. Wang (University of Alberta), R. Bartus (Cortex Pharmaceuticals); R. Green (University of Illinois)

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Drug Receptor Interactions

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

2.3

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The extracellular adenosine receptor has a modulatory role in the nervous, circulatory, endocrine, and immunological systems. The prospect of harnessing these effects specifically for therapeutic purposes is attractive.

A functionalized congener approach to drug design has been applied to the adenosine receptor to produce analogs of agonists and antagonists which have promise as therapeutic agents and as receptor probes. Prodrugs of adenosine agonists and antagonists targeted for the brain and kidneys are being designed. In the antagonist series new analogs which combine potency, water solubility, and  $A_1$ -adenosine receptor selectivity in the same compound are now being evaluated in in vivo testing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 DK 19612-01 LC
--	--

PERIOD COVERED  
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Aldose Reductase Inhibitors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)  
PI: Louis A. Cohen Chief, Section on Biochem. Mech. NIDDK-LC  
  
Others: Rita Labroo Special Volunteer GWU

COOPERATING UNITS (if any)  
Peter Kador, LMOD, NEI

LAB/BRANCH  
Laboratory of Chemistry

SECTION  
Section on Biochemical Mechanisms

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS 0.2	PROFESSIONAL 0.2	OTHER
------------------------	---------------------	-------

CHECK APPROPRIATE BOX(ES)  
☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Inhibition of the enzyme, aldose reductase, represents a new pharmacological approach toward the treatment of late-onset diabetic complications. These complications affect the eye (cataracts), kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of high intracellular concentrations of sorbitol, in turn resulting from the reduction of excess glucose symptomatic of diabetes. Aldose reductase is the enzyme responsible for sorbitol formation.

Our studies on the synthesis of inhibitors of tryptophan-metabolizing enzymes have produced oxindole derivatives which are somewhat analogous in overall geometry to certain commercial compounds now in advanced clinical trials as aldose reductase inhibitors. In vitro assays have revealed that, while these oxindole derivatives (spirolactones) are not significantly active, their hydrolysis products are as potent as any others thus far designed. Although these chiral compounds are not amino acids, we have succeeded in the use of chymotrypsin to achieve their resolution by selective ester hydrolysis. The introduction of fluorine on the benzene ring of the oxindole has been found to increase inhibitor activity 2-3 fold. Additional analogues have been prepared, which are expected to serve as irreversible affinity and photoaffinity labels for the enzyme. Current efforts are being devoted to improvements in synthesis yields, in resolution and in enhancement of lipophilicity, with the goal of achieving more effective penetration and transport to the sites of action in vivo.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19613-01 LC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Virender M. Labroo Special Expert NIDDK-LC

Others: Albinus D'Sa Visiting Fellow NIDDK-LC

Louis A. Cohen Chief, Section on Biochem. Mech. NIDDK-LC

## COOPERATING UNITS (if any)

J. L. Flippen-Anderson, Naval Research Laboratory, Washington, DC; W. Dürckheimer, FRG

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

1.5

## PROFESSIONAL

1.4

## OTHER

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Introduction of conformational restraints in biologically active peptides is a very useful approach to probe peptide-receptor interactions and to enhance their potency and/or selectivity. While local conformational constraints can be imposed by incorporating dehydro- or cyclopropane amino acids, side chain-side chain cyclization to form cyclic analogues has been found to be one of the most useful approaches for introducing general conformational constraints. The use of disulfide bond between Cys or Pen residues or amide bond between side chains of amino and carboxy trifunctional amino acids. The 13-membered cyclic peptide, H-Tyr-D-Orn-Phe-Asp-NH<sub>2</sub>, is a potent  $\mu$ -selective opioid peptide. Molecular modeling of this peptide has revealed considerable flexibility in the ring structure, making it difficult to identify the possible biologically relevant conformation. In order to restrict further the conformational mobility in such peptides, we have designed a novel trifunctional amino acid,  $\beta$ -(2-pyrrolidinyl)alanine (PDA). This amino acid, if used in place of Orn or Lys for side chain-side chain cyclization, is expected to reduce considerably the conformational flexibility in the macrocyclic ring of these peptides owing to the introduction of a bicyclic structure. Moreover, the additional asymmetric center in the pyrrolidine ring may introduce differential stereochemical constraints to the binding of diastereoisomeric peptides, derived from the corresponding diastereoisomeric PDAS. Thus, these peptides may act as probes for delineating the stereochemical topology of the receptor in the vicinity of the ring structure and may have interesting biological activities.

Optically active amino acids are being used increasingly as synthons for preparation of a variety of chiral compounds. PDAS can be expected to be excellent synthons for compounds such as pyrrolizidines with the stereochemistry already defined at two centers. Furthermore, these amino acids may possess antimicrobial and/ or antimetabolic activities. An efficient method for the synthesis of all the four optically active stereoisomers of PDA has been developed. These amino acids will be used for the preparation of novel bicyclic opioid peptide analogues.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE	PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 DK 19614-01 LC

PERIOD COVERED  
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Fluorinated Analogues of Bioactive Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Virender M. Labroo	Special Expert	NIDDK-LC
Others:	Louis A. Cohen	Chief, Section on Biochem.	
		Mech.	NIDDK-LC
	David Hebel	Visiting Fellow	NIDDK-LC
	Kenneth L. Kirk	Chief, Section on Drug Receptor Interactions	NIDDK-LC

COOPERATING UNITS (if any)  
P. W. Schiller, Montreal, Canada; W. Dürckheimer, Frankfurt, FRG

LAB/BRANCH  
Laboratory of Chemistry

SECTION  
Section on Biochemical Mechanisms

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS 0.3	PROFESSIONAL: 0.3	OTHER:
------------------------	----------------------	--------

CHECK APPROPRIATE BOX(ES)  
☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Fluorinated analogues of a wide range of structurally different classes of organic compounds have been prepared and many of these have proven to be important pharmacological and medicinal agents. The advantages of fluorine substitution in large part arise from the small van der Waals radius of fluorine (1.35 Å<sup>o</sup>). As a result, fluorine bonded to an sp<sup>2</sup>-carbon is used effectively as a C-H replacement. Furthermore, because of similar aliphatic C-F and C-O bond lengths (1.39 Å<sup>o</sup> and 1.43 Å<sup>o</sup>, respectively) and because of the weak hydrogen bond- accepting property of the former, fluorine bonded to an sp<sup>3</sup>-carbon has been used frequently as an OH surrogate, particularly in carbohydrate research. Thus, because of steric and some electronic similarities, fluorinated compounds often mimic their nonfluorinated parents with respect to recognition by biological macromolecular systems such as enzymes, transport proteins and receptors. On the other hand, the high electronegativity of fluorine (4.0) can drastically alter electron density distribution in the molecule which, in turn, affects pK<sub>a</sub>'s of neighboring functional groups and molecular dipole moments. All these altered physico-chemical properties of the molecule, as a result of fluorine substitution, can result in drastically modified biological properties such as potency and receptor selectivity. Furthermore, recent advances in <sup>19</sup>F-NMR and electron energy-loss spectroscopic techniques, and <sup>18</sup>F-positron emission tomography (<sup>18</sup>F-PET) have increased the significance of fluorinated molecules as biological markers.

Although many methods have been developed for fast and efficient incorporation of <sup>18</sup>F and <sup>19</sup>F into various compounds of medicinal importance, such methods in general have not been practical for direct incorporation into peptides. We have now developed a method for fast, efficient introduction of F regiospecifically into the phenolic ring of Tyr-containing peptides by use of electrophilic fluorinating agent acetyl hypofluorite (AcOF). We have used this method for fluorinating μ-selective opioid peptides. In vitro bioassays and receptor binding assays have revealed that these fluoro peptides retain their high receptor selectivity. Thus we have been able to develop a very μ-selective (K<sub>i</sub><sup>0</sup>/K<sub>d</sub><sup>0</sup> = 5390) fluoro peptide [Tyr(3-F)-D-Arg-Phe-Lys-NH<sub>2</sub>] which may be used as an useful research tool to probe the complex opioid receptor system.



ANNUAL REPORT OF THE LABORATORY OF CELL BIOLOGY AND GENETICS  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Cell Biology and Genetics carries on a broad program of investigation into exocrine and endocrine secretion and the molecular events regulating these processes. Four specific tissues have been studied: chromaffin cells, which secrete adrenaline, ATP and endogenous opiates; pancreatic beta cells, which secrete insulin; nerve terminals which secrete transmitters and colon carcinoma cells which secrete mucins. The following studies have been performed in pursuit of these goals.

I. MOLECULAR BIOLOGY AND BIOPHYSICS OF THE SECRETORY PROCESS IN CHROMAFFIN CELLS.

A. Calcium channel activity of purified human synexin and structure of the human synexin gene

Synexin is a calcium-dependent membrane binding protein that not only fuses membranes but also acts as a voltage-dependent calcium channel. We have isolated and sequenced a set of overlapping cDNA clones for human synexin. The derived amino acid sequence of synexin reveals strong homology in the C-terminal domain with a previously identified class of calcium-dependent membrane binding proteins. These include endonexin II, lipocortin I, calpactin I heavy chain (p36), protein II, and calelectrin 67K. The  $M_r$  51,000 synexin molecule can be divided into a unique, highly hydrophobic N-terminal domain of 167 amino acids and a conserved C-terminal region of 299 amino acids. The latter domain is composed of alternating hydrophobic and hydrophilic segments. Analysis of the entire structure reveals possible insights into such diverse properties as voltage-sensitive calcium channel activity, ion selectivity, affinity for phospholipids, and membrane fusion. We suggest that the conserved C-terminal domain in the locus of the channel activity, and that the highly hydrophobic N-terminal domain is involved in forming the 'hydrophobic bridge, which we have previously postulated as the mediator of fusion between two adjacent membranes.

B. Synexin (Annexin 7) Polymorphisms and Expression in E. coli

Synexin is a calcium-dependent membrane binding protein that aggregates chromaffin granules and acts as a voltage-dependent calcium channel in vitro. DNA sequencing of human synexin cDNAs from five libraries (B-cell, fibroblast, liver, lung, and retina) reveals that synexin contains the conserved C-terminal region common to all annexins, but is unique in having a long, hydrophobic N-terminus of 167 amino acids (synexin A or annexin 7A). Also, sequence results from several clones indicate that the synexin RNA is being alternatively spliced and polyadenylated. One cDNA has a cassette exon of 66 base pairs (bp) inserted in the 5' end. This mRNA is the predominant synexin transcript in brain and muscle (skeletal and cardiac) tissues and results in the synthesis of a synexin isoform (synexin B or annexin 7B) with three out of twenty-two inserted amino acids being acidic. Additional data to support this conclusion include the analysis of (1) mRNA using PCR, (2) human skeletal muscle on Westerns, and (3) sequences of genomic splice junctions. The second polymorphism results in a 336 bp longer mRNA due to the selection of a

more 3' polyadenylation signal and accounts for the two bands seen after autoradiography of Northern blots of human RNA. In addition, human synexin expressed in *E. coli* with the pTrc vector has similar *in vitro* activities as human liver synexin described above. Several different constructs of synexin and endonexin (annexin 5) also synthesize in *E. coli* immunoreactive proteins with the appropriate molecular weights. These recombinant proteins are currently being analyzed.

#### C. Cloning and Sequencing of the Human Nucleolin cDNA

Nucleolin is a nucleolar protein important for the maturation of ribosomal RNA. A cDNA containing the entire coding region for human nucleolin has been isolated from a  $\lambda$ gt10 human retinal library using a bovine cDNA probe. The cDNA hybridized to a transcript of 3000 bases from fast-dividing cells, as well as terminally differentiated tissues of several species. Translation of the nucleotide sequence revealed a long open reading frame which predicts a 707 amino acid protein with several distinct domains. These include repeating elements, four conserved RNA-binding regions, a glycine-rich carboxy-terminal domain and sites for phosphorylation, glycosylation and dibasic cleavage. Human and bovine nucleolin exhibited more additions and/or substitutions of aspartate, glutamate and serine residues in the chromatin-binding domains by comparison with the hamster and mouse nucleolins. These differences may be related to species-specific functions in transcription.

#### D. Kinetic Characteristics of Calcium-dependent, Cholinergic Receptor Controlled ATP Secretion from Adrenal Medullary Chromaffin Cells

Adrenal chromaffin cells secrete catecholamines (CA) and ATP in response of acetylcholine (ACh) and high  $[K^+]_o$ . The release process is relatively fast making it difficult to measure the early phase of the secretory response. Recently we were able to resolve the time course of the secretory response by measuring the release of ATP using luciferin luciferase included in the extracellular medium. For the three secretagogues studies, ACh, nicotine and high  $[K^+]_o$ , the early phase of release followed a complex kinetics. Allowing for an initial delay of the secretory response, the kinetics could be described as the sum of two power exponential processes. Increasing the temperature from 23 to 37 C induced a marked decrease in the two time constants needed to fit the early time course of the ATP secretion. The activation energies, estimated from Arrhenius plots, were approx. 20 and 16 kcal/mol for both phases of ATP release induced by either cholinergic agonists or high  $[K^+]_o$ . These results suggest that cholinergic receptor activation and membrane depolarization induce ATP (and CA) secretion through a common pathway. The initial delay in the onset of the secretory response decreased with increasing doses of secretagogue and with temperature. We propose that the delay preceding the actual onset of ATP release represents the time required for generation of intracellular second messengers. The effective concentration attained by these messengers depend apparently on both receptor occupancy by the agonist and the ensuing  $Ca^{2+}$  channel activation.

#### E. Purification and Properties of a Barium-dependent Chromaffin Granule Aggregating Protein from Bovine Tissues

Barium ions can enter chromaffin cells and induce secretion by a mechanism independent of calcium ions (J. Biol. Chem., 264:7914-7920, 1989). We

have previously suggested that synexin might be responsible for calcium-dependent exocytosis. However, since synexin reacts exclusively with calcium, we searched for a protein present in chromaffin cells which should be able to aggregate chromaffin granules in the presence of barium ions. Here we report the purification of such a protein from chromaffin cells and other bovine tissues, and describe some of its more salient properties. We were able to purify this protein by conventional and FPLC methods, and found it to be active in the pure state (MW=37000 Da.) with either barium ( $K = 350\mu\text{M}$ ) or calcium ( $K = 40\mu\text{M}$ ). It does not crossreact with antibodies to synexin. The barium-dependent granule aggregation activity has a Hill coefficient ( $n_H$ ) of 6.0 in terms of protein concentration ( $K = 30\text{nM}$ ). However, activation by either calcium or barium has a simple hyperbolic dependence and the maximal aggregation is the same for both cations. The phenothiazine drugs trifluoperazine and promethazine block the aggregating activity of the protein. We have also tested this protein for fusion ability by following mixing of internal volumes or the mixing of lipids in large unilamellar liposomes. We found that this protein enhances aggregation and fusion of liposomes in the presence of either  $\text{Ba}^{2+}$  or  $\text{Ca}^{2+}$ . We conclude that this protein is a candidate for the sought after barium-dependent fusion protein in chromaffin cells, and that its properties are consistent with properties predicted from kinetic studies of secreting cells.

F.  $\text{Ba}^{2+}$ -Induced ATP Release From Adrenal Medullary Chromaffin Cells is Mediated By  $\text{Ca}^{2+}$  Entry Through Both Voltage- and Receptor-Gated  $\text{Ca}^{2+}$  Channels

Barium is a classical secretagogue for chromaffin cells, although the details of how it evokes release are yet to be fully understood. We have measured on-line the exocytotic secretion of ATP from adrenal medullary chromaffin cells induced by  $\text{Ba}^{2+}$  using a luciferin/luciferase assay. We have found that  $\text{Ba}^{2+}$ -induced ATP release requires the entry of  $\text{Ba}^{2+}$  through either voltage or receptor-gated  $\text{Ca}^{2+}$  channels. This conclusion is based on observations that short preincubations with low concentrations of either nicotine or  $\text{K}^+$  greatly enhance  $\text{Ba}^{2+}$ -induced ATP release and that this augmentation can be blocked with the nicotinic receptor antagonist, hexamethonium, and the  $\text{Ca}^{2+}$  antagonist, nifedipine, respectively. Moreover, both nicotine and  $\text{K}^+$  stimulate  $\text{Ba}^{2+}$  uptake, which in the case of  $\text{K}^+$  is inhibited by nifedipine. These results support the hypothesis that the cellular events leading to  $\text{Ba}^{2+}$ -induced secretion coincide at least in part with the events leading to  $\text{Ca}^{2+}$ -dependent exocytosis.

G. Voltage-Sensitive Calcium Flux Into Bovine Chromaffin Cells Occurs Through Dihydropyridine-Sensitive And Dihydropyridine And  $\omega$ -Conotoxin-Insensitive Pathways

The fluorescent  $\text{Ca}^{2+}$ -indicator FURA-2 was used to characterize the depolarization-related intracellular  $\text{Ca}^{2+}$  signalling process in bovine adrenal chromaffin cells. Depolarization with high  $\text{K}^+$  (10-65 mM) gave rise to a very rapid increase in intracellular free  $\text{Ca}^{2+}$  concentration, which subsequently decayed slowly towards a "plateau."

The size of this initial increase varied sigmoidally with the calculated membrane potential, the relationship being described well by a Boltzmann distribution function for a transition between two states (transition potential,  $-23\text{mV}$ ). A dihydropyridine calcium channel agonist [(+)-202-791,  $1\mu\text{M}$ ] raised intracellular free  $\text{Ca}^{2+}$  concentration further in the presence of  $30\text{ mM K}^+$ , and it enhanced the initial intracellular  $\text{Ca}^{2+}$  response to depolarization. Voltage-sensitive calcium channels in chromaffin cells are believed to include the L-type. Several dihydropyridine calcium channel antagonists [(-)-202-791, nifedipine, nitrendipine;  $1-5\mu\text{M}$ ], known to be active on L-type channels, caused only modest inhibition of  $\text{K}^+$ -induced increase in intracellular free  $\text{Ca}^{2+}$  concentration: c. 50% (at  $30\text{ mM K}^+$ ) and 25% (at  $40-70\text{ mM K}^+$ ). In addition,  $\omega$ -conotoxin GVIA ( $1-10\mu\text{M}$ ), a blocker of neuronal N- and L-type calcium channels, reduced the initial increase in intracellular free  $\text{Ca}^{2+}$  concentration only slightly at  $55\text{ mM K}^+$ . Further, the dihydropyridine-insensitive component of the intracellular  $\text{Ca}^{2+}$  signal was also insensitive to  $\omega$ -conotoxin, which was otherwise quite active in a central nervous rat in vivo preparation.  $\text{Gd}^{3+}$  ( $40\mu\text{M}$ ), a potent calcium antagonist in the chromaffin cell, blocked the intracellular  $\text{Ca}^{2+}$  response to depolarization. When added at different times after  $\text{K}^+$  stimulation, however,  $\text{Gd}^{3+}$  reduced intracellular free  $\text{Ca}^{2+}$  concentration to control levels along a slow time course of several minutes. Similar results were obtained when EGTA was added to reduce extracellular  $\text{Ca}^{2+}$  concentration to sub-nanomolar levels, in the presence of high  $\text{K}^+$ . We conclude that bovine chromaffin cells were equipped with at least two different classes of voltage-dependent calcium channels, only one of which is likely to be the L-type channel. We also propose that depolarization, in addition to stimulating  $\text{Ca}^{2+}$  influx, may also lead to enhancement to  $\text{Ca}^{2+}$  release from an intracellular store.

#### H. Effects of Calcium and Bay K-8644 on Calcium Currents in Adrenal Medullary Chromaffin Cells

The kinetic and steady-state characteristics of calcium currents in cultured bovine adrenal chromaffin cells were analyzed by the patch-clamp technique. Whole cell inward  $\text{Ca}^{2+}$  currents, recorded in the presence of either  $5.2$  or  $2.6\text{ mM Ca}^{2+}$  exhibited a single, nor inactivating component. To analyze the effects of  $\text{Ca}^{2+}$  and Bay K-8644 on the kinetics of the  $\text{Ca}^{2+}$  currents, we used a modified version of the Hodgkin-Huxley empirical model. At physiological  $[\text{Ca}^{2+}]$  ( $2.5\text{ mM}$ ) the midpoint of the steady-state  $\text{Ca}^{2+}$ -channel activation curve lay at  $-6.9\text{ mV}$ . Increasing the  $[\text{Ca}^{2+}]_0$  to  $5.2\text{ mM}$  shifted the midpoint by  $-4.3\text{ mV}$  along the voltage axis. At the midpoint, changes in potential of  $7.8\text{ mV}$  (for  $5.2\text{ mM Ca}^{2+}$ ) and  $9.2\text{ mV}$  (for  $2.5\text{ mM Ca}^{2+}$ ) induced an e-fold change in the activation of the current. Increasing  $[\text{Ca}^{2+}]_0$  from  $2.5$  to  $5.2\text{ mM}$  induced a marked increase in the rate constant for turning on the  $\text{Ca}^{2+}$  permeability. Conductances were estimated from the slope of the linear part of the current-voltage relationships as  $8.7$  and  $4.2\text{ nS}$  in the presence of  $5.2$  and  $2.5\text{ mM Ca}^{2+}$ , respectively. Incubation of the cells in the presence of Bay K-8644 at increasing concentrations from  $0.001$  to  $0.1\mu\text{M}$  increased the slope conductance from  $4.2$  to  $9.6\text{ nS}$ . Further increases in the concentration of Bay K-8644 from  $1$  to  $100\mu\text{M}$  induced a marked reduction in the conductance to  $1.1\text{ nS}$ .

In the presence of Bay K-8644 (0.1  $\mu$ M) the midpoint of the activation curve was shifted by 6.1 mV towards more negative potentials, i.e., from -6.9 to -13 mV. At the midpoint potential of -13 mV, a change in potential of 6.9 mV caused an e-fold change in  $\text{Ca}^{2+}$  permeability. The kinetic analysis showed that Bay K-8644 significantly reduced the size of the rate constant for turning off the  $\text{Ca}^{2+}$  permeability.

#### I. Interaction of Protein Kinase C with Chromaffin Granule Membranes: Effects of $\text{Ca}^{2+}$ , Phorbol Esters and Temperature Reveal Differences in the Properties of the Association and Dissociation Events

Interaction of protein kinase C with chromaffin granule membranes has been studied as a means of investigating the membrane surfaces, which is believed to occur during secretion. Protein kinase C in an adrenal medullary soluble fraction was found to bind reversibly to granule membranes in a  $\text{Ca}^{2+}$ -dependent fashion. Association and dissociation events were sensitive to  $\text{Ca}^{2+}$  concentrations in the low micromolar range, and the  $\text{Ca}^{2+}$  sensitivity of both processes was increased when the membranes had been preincubated with protein kinase C activating phorbol ester, 4 $\beta$ -phorbol 12 myristate 13-acetate (TPA). Binding of protein kinase C to granule membranes occurred at 0 and 37°C, irrespective of whether the membranes had been preincubated with with TPA. However, dissociation of protein kinase C, from granule membranes that had been preincubated with TPA occurred only at 37°C and not at 0°C. These effects of TPA were not reproduced by 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ PDD), a phorbol ester which does not activate protein kinase C. Soluble protein kinase C activity also associated with chromaffin granules in a  $\text{Ca}^{2+}$ -dependent manner in an adrenal medullary homogenate, indicating that granules can compete with other intracellular membranes for the binding of protein kinase C. Results obtained with this model system differ from other systems where the interaction of protein kinase C with plasma membranes has been studied and have general implications for studies performed on the translocation of protein kinase C in intact cells and for the role of protein kinase C in stimulus-secretion coupling in the chromaffin cell.

#### J. Barium Induces Secretion From Chromaffin Cells By A Mechanism Independent of Calcium

Barium ions enter chromaffin cells via voltage-sensitive calcium channels, although the intracellular site of barium action is distinct from that of calcium. The entry of barium primarily through possessed functional voltage-sensitive channels was indicated by experiments showing inhibition of  $^{135}\text{Ba}^{2+}$  uptake was stimulated by 50 mM KCl but not by nicotine. Furthermore,  $^{133}\text{Ba}^{2+}$  uptake was inhibited by hyperosmolarity, which specifically blocks the voltage-sensitive calcium channel but not the receptor-associated calcium channel. These conclusions from studies on barium uptake were also borne out by experiments measuring catecholamine secretion. Thus, blockers of voltage-dependent calcium channels which inhibited barium uptake also inhibited barium-induced catecholamine secretion. In other experiments, simultaneous stimulation with nicotine and barium in the presence of calcium evoked coincident and additive catecholamine secretion. By contrast, when 50 mM KCl was substituted for nicotine in the same experimental design, barium ions inhibited potassium-induced catecholamine secretion at low calcium concentrations. Only at high calcium concentrations were barium-induced and potassium induced secretion additive. These data also

indicate that barium and calcium compete at the voltage-sensitive not the receptor pathway. Furthermore, these additivity data suggest that once inside the cell, barium and calcium have two distinct mechanisms of action. As predicted by this hypothesis, in digitonin-permeabilized chromaffin cells either calcium or barium stimulated catecholamine release; and in the presence of both cations catecholamine secretion was equivalent to the sum of secretion with either cation alone. Additional support for this concept comes from experiments showing that while calcium mediated catecholamine secretion is sensitive to trifluoperazine and imipramine, barium-mediated secretion is not. Taken together, all these data indicate that there are two distinct intracellular sites of action for barium and calcium. In contrast to catecholamine secretion, nonexocytotic ascorbic acid secretion was induced by nicotine and potassium in the presence of calcium, but not by barium alone. These data provide additional evidence that barium acts by a different mechanism than calcium, in still another secretory system in chromaffin cells.

K. The Muscarinic Agonist Oxotremorine-M Activates Catecholamine Secretion and  $^{45}\text{Ca}^{++}$  Uptake In Bovine Chromaffin Cells: Evidence For A Novel Type of Cholinergic Receptor

Bovine chromaffin cells secrete catecholamines (CA) in response to nicotinic but not muscarinic agonists. Chromaffin cells from most other species, however, secrete in response to muscarinic agonists, and it puzzled us that while bovine cells possessed functional muscarinic receptors they were nonetheless not coupled directly to secretion. To examine this in more detail we tested the effect of a series of muscarinic agonists on secretion and  $^{45}\text{Ca}^{++}$  uptake in bovine chromaffin cells. We found that the full muscarinic agonist oxotremorine-M (Oxo-M) induced a robust CA secretion. Oxotremorine evoked only a moderate secretion at the highest concentration tested (1mM). By contrast, muscarine, pilocarpine, bethanechol, and McN-A-343 did not elicit any secretory response. Secretion by both Oxo-M and nicotine was inhibited by hexamethonium and high concentrations of atropine (100 $\mu\text{M}$ ). Secretion induced by nicotine and Oxo-M but not by high  $\text{K}^+$  was blocked by the selective ganglionic M-1 agonist, McN-A-343, indicating a direct effect on the secretory mechanism. Secretion induced by Oxo-M and nicotine was also relatively insensitive to both kappa bungarotoxin, an effective ganglionic nicotinic blocker, and to alpha bungarotoxin, a specific blocker of muscle nicotonic receptors. Pretreatment with either nicotine or Oxo-M led to desensitization to a repeated dose of either agonist. However, homologous desensitization was more profound. Oxo-M also induced  $^{45}\text{Ca}^{++}$  uptake by chromaffin cells. These data suggest that the "nicotinic" receptor in bovine chromaffin cells may have some muscarinic character, and is distinct from those nicotinic receptors heretofore described in ganglia, brain or muscle. We have tentatively named this unusual receptor the "muscatinic receptor."

L. Monoclonal Antibodies Against the Presynaptic Calcium Channel Antagonist  $\omega$ -conotoxin GVI A from Cone Snail Poison

Monoclonal antibodies have been prepared against  $\omega$ -conotoxin GVI A, a peptide isolated from marine snails of the genus *Conus* (*Conus geographus* and *Conus magus*).  $\omega$ -Conotoxin GVIA ( $\omega$ -ctx GVIA) is a bicyclic 27 amino acid peptide isolated from the marine snail, *Conus geographus* and *Conus magus* [1,2], which inactivates select presynaptic calcium channels in the central nervous system [3,6]. The bicyclic structure is defined by disulfide bonds between 6 cysteines. This toxin is a blocker of select presynaptic  $\text{Ca}^{2+}$  channels in the central nervous system. Antigenic conotoxin GVI A was synthesized as a covalent conjugate with bovine serum albumin and injected s.c. An ELISA assay combined with a competitive inhibition assay was used to select and characterize monoclonal antibodies able to recognize and bind the free toxin. Several of the antibodies were found to block  $\omega$ -conotoxin GVI A inhibition of  $^{45}\text{Ca}$  transport into rat brain synaptosomes and to block  $\omega$ -conotoxin GVI A binding to membranes from the same preparation. The antibodies recognize native, synthetic toxin, and are useful for analysis of toxin in biological fluids.

II. BIOPHYSICS OF INSULIN SECRETION FROM BETA CELLS IN ISLETS OF LANGERHANS

A. Muscarinic Receptor Modulation of Glucose-induced Electrical Activity in Mouse Pancreatic B-cells

Acetylcholine (1-10 $\mu\text{M}$ ) depolarized the membrane and stimulated glucose-induced bursts of electrical activity in mouse pancreatic B-cells. The acetylcholine effects were mimicked by muscarine while nicotine had no effect on membrane potential. Pirenzepine, an antagonist of the classical M-1 type muscarinic receptors, but not gallamine (1-100 $\mu\text{M}$ ), an antagonist of the classical M2-type receptors, antagonized the acetylcholine action on glucose-induced electrical activity ( $\text{IC}_{50}=0.25\mu\text{M}$ ). Bethanechol, an agonist of the classical M2-type muscarinic receptors, was approximately 100 times less effective than acetylcholine in stimulating the electrical activity. In addition, acetylcholine (1 $\mu\text{M}$ ) induced a marked increase (25%) input resistance to the B-cell membrane. The results indicate that acetylcholine exerted its effects on the B cell membrane by inhibiting  $\text{K}^{+}$  conductance via activation of the muscarinic receptor subtype distinct from the classical M2-type receptor.

B. A New Class of Calcium Channels Activated By Glucose In Human Pancreatic  $\beta$ -Cells.

Single calcium-channel currents were recorded from membrane patches of cultured  $\beta$ -cells dissociated from human islets of Langerhans. In the absence of exogenous glucose, low frequency spontaneous calcium-channel

openings of small amplitude ( $-0.34 \pm 0.02$  pA at ) mV pipet potential) were observed in all membrane patches examined (25 mM  $\text{Ca}^{2+}$  in the patch pipet). The frequency of channel openings was rather insensitive to the membrane potential across the patch (range from ca 0 to 60 mV pipet potential; chord conductance  $4.9 \pm 0.2$  pS). Addition of glucose induced a dose-dependent increase in the frequency of openings of the  $\text{Ca}^{2+}$ -channel (from now on referred to as the  $\text{Ca}_G$ -channel). A few minutes after the addition of glucose ( $\geq 11$  mM), burst of action potentials were often observed which were elicited only if  $\text{Ca}^{2+}$  was present in the solution bathing the  $\beta$ -cells. Application of glucose in the presence of mannoheptulose (11 mM), a blocker of the hexokinase controlling the first stage of glycolysis, had no effect and the activity of the  $\text{Ca}_G$ -channel remained at its resting level. The readily permeant mitochondrial substrate 2-ketoisocaproate (KIC, 10 mM) was as effective as glucose in eliciting action potentials from cell forming part of cell aggregates. The activity of the  $\text{Ca}_G$ -channel was significantly increased by KIC (11 mM). Although spike and  $\text{Ca}^{2+}$ -channel activity were markedly stimulated by glucose or KIC in all cells examined, regular bursts of action potentials were seen only if the patch was formed on  $\beta$ -cells which were part of a cell aggregate. Mannoheptulose (11 mM) prevented the activation of the  $\text{Ca}_G$ -channel by glucose (11 mM) but not by KIC 11 mM). Once activated, the  $\text{Ca}_G$ -channel remained active even after excision of the patch. We propose that the physiological control of this new  $\text{Ca}^{2+}$ -channel is mediated by one or more products of glucose metabolism.

#### C. Quinine Blocks High Conductance, Calcium-activated Potassium Channel in Rat Pancreatic- $\beta$ -Cells

The  $[\text{Ca}^{2+}]_i$ -activated  $\text{K}^+$ -channel, one of the 3  $\text{K}^+$ -channels described in pancreatic  $\beta$ -cells, is a high conductance, voltage-dependent  $\text{K}^+$ -channel. Quinine, known to block  $[\text{Ca}^{2+}]_i$ -activated  $\text{K}^+$ -channels in other cells, has been described to block the silent phase between the bursts of glucose evoked electrical activity in mouse pancreatic  $\beta$ -cells, and to inhibit  $\text{K}^+$  efflux from rat pancreatic islets. We report here that quinine blocks the  $[\text{Ca}^{2+}]_i$ -activated  $\text{K}^+$ -channel in rat pancreatic  $\beta$ -cells from the external side of the membrane. We also show that the blockade is characterized by fast flickering of the  $\text{K}^+$ -channel between the open and closed state. Mean open and closed times within bursts were found to be exponentially distributed, suggesting that the blockade by quinine involves obstruction of the  $\text{K}^+$  flow through the open channel.



D. Modulation of the Frequency of Glucose-Dependent Bursts of Electrical Activity By  $\text{HCO}_3/\text{CO}_2$  in Rodent Pancreatic Experimental and Theoretical Results

The burst pattern of electrical activity recorded from pancreatic  $\beta$ -cells in response to 11 mM glucose shows a large islet to islet variability. The relationship between burst frequency and glucose sensing (the threshold for electrical activity and the graded increase in electrical response to glucose, i.e., active phase %) has not been investigated within the same islet. In this work, we show that low  $\text{HCO}_3$  (5 mM) Hepes buffered solutions reversibly reduce the frequency of bursts compared to control (25 mM)  $\text{HCO}_3$  buffered solutions in the same islet. There was no change in the threshold or active phase (%). Using the mathematical model of Sherman et al. 1988, we explored mechanisms for a change in frequency independent of a change in active phase (%). Increased exchangeable calcium pool size and increased cell to cell coupling were the two theoretical treatments which could reproduce the experimental data. We conclude that burst frequency can be modulated independent of the active phase and that alteration of a calcium pool size best fits the experimental data.

III. BIOPHYSICAL AND TOXICOLOGICAL ANALYSIS OF NEUROTRANSMISSION AND NEURAL DAMAGE

A. Characteristics of Two Types of Calcium Channels In Rat Pituitary Gonadotrophs

The properties of  $\text{Ca}^{2+}$  channels in cultured rat pituitary gonadotrophs were analyzed by the patch-clamp technique. The inward  $\text{Ca}^{2+}$  currents, recorded in the presence of 5.2 mM  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ , included a fast transient component with activation-inactivation kinetics and a delayed component with slower activation. The midpoint of the activation curve lay at -30 mV for the transient component and at -12 mV for the delayed component. At the midpoint, changes in potential of 9.5 and 13 mV induced an e-fold change in the activation of the transient and delayed component respectively. The rate of inactivation of the first component was strongly voltage dependent. At -43 mV, a 7.4 mV change in potential induced an e-fold change in the fractions of calcium channels available to conduct  $\text{Ca}^{2+}$ -current. During long-lasting (100-200 ms) low frequency depolarizing voltage-clamp pulses, the size of the delayed component of the  $\text{Ca}^{2+}$  current remained constant. The differential effects of membrane potential on inactivation and the different time constants for activation of the two components of the  $\text{Ca}^{2+}$  conductance indicate the presence of two types of  $\text{Ca}^{2+}$  channels in the membrane of the gonadotroph: the rapidly inactivating current appears to be attributable to a T-type channel, and the noninactivating current corresponds to the L-type channel described in many other cell types.

B. Desensitization of Pituitary Gonadotropin Secretion By Agonist-Induced Inactivation of Voltage-Sensitive Calcium Channels

Gonadotropin-releasing hormone (GnRH) stimulates calcium mobilization and influx in pituitary gonadotrophs, and agonist induced calcium entry through voltage-sensitive channels (VSCC) is required for the maintenance of gonadotropin secretion. However, prolonged or frequent exposure to GnRH attenuates the extracellular  $\text{Ca}^{2+}$ -dependent cytosolic  $\text{Ca}^{2+}$  signal and diminishes hormone secretion. Measurements of membrane  $\text{Ca}^{2+}$  currents revealed significant impairment of VSCC activity in gonadotrophs during desensitization by GnRH. VSCC were also inactivated in a calcium-dependent manner during exposure to high  $\text{K}^+$ . Prolonged inactivation of such  $\text{Ca}^{2+}$  channels by high  $\text{K}^+$  reduced the calcium and secretory responses to GnRH and vice versa. The calcium-dependent inactivation of VSCC during GnRH action appears to be a primary factor in the onset of desensitization in pituitary gonadotrophs. This mechanism could also account for the development of agonist-induced refractoriness in other calcium-regulated target cells.

C. Distribution of a Putative Kainic Acid Receptor In Nervous System Determined with Monoclonal and Antibodies: Evidence for Synaptic and Extrasynaptic Sites

A frog brain kainic acid receptor (KAR) was studied using monoclonal and polyclonal antibodies against the affinity purified receptor. Immunocytochemistry was done on sections of the frog CNS, and the distribution of high- and low-affinity  $^3\text{H}$ -kainic acid ( $^3\text{H}$ -KA) binding sites determined with in vitro receptor autoradiography. These studies showed (1) similar distributions of high- and low-affinity  $^3\text{HKA}$  binding sites, (2) identical patterns of immunostaining with the polyclonal antibodies and 2 monoclonal antibodies, and (3) an antibody binding distribution which closely matched that of  $^3\text{H}$ -KA binding, suggesting that the antibodies recognize the primary KAR in frog brain. In the frog brain, an anteroposterior gradient of immunostaining was observed, with the telencephalon intensely and uniformly immunoreactive. Other areas intensely immunoreactive included the cerebellum, the infundibulum, the tectal and posterior commissures, and the laminar nucleus of the torus semicircularis. The optic tectum showed selective staining of the plexiform layers 3 and 5-7. The pattern of staining was punctate and appeared to be associated with nerve fibers, among them dendritic arborizations. Electron microscopic observation showed staining at the cytoplasmic side of postsynaptic membranes. Extrasynaptic staining was observed as patches on the surface of unmyelinated nerve processes.

D. Characterization and Localization of Cell Binding Sites  
Biotinylated Tetanus Toxin on Nerve Growth Factor-Treated PC12 Cells

Biotinylated derivatives of tetanus toxin were prepared and isolated by chromatofocusing and ganglioside-affinity chromatography. Biotinylation was monitored by the appearance of a 210,000 dalton complex upon SDS-polyacrylamide gel electrophoresis in the presence of avidin, and by selective binding to an avidin-Sepharose gel. At molar biotin-toxin ratios from 1:1 to 20:1 only biotinylated derivatives with low toxicity were obtained; these derivatives, however, retained 60-80% of their specific binding affinity for brain synaptosomes. A biotinylated tetanus toxin derivative purified by ganglioside-affinity chromatography was used to identify and localize tetanus toxin binding sites on PC12 cells. Electron microscopic analysis with streptavidin-gold revealed very low levels of tetanus toxin binding sites on the surface of untreated cells, and the appearance of such binding sites during the second week of nerve growth factor-induced differentiation. Examination of micrographs of the differentiated cells indicated that the tetanus toxin binding sites are concentrated on the neurites, with relatively few appearing on the cell bodies. Cognate studies using  $^{125}\text{I}$  labelled, affinity-purified tetanus toxin revealed an increase in PC12 binding capacity from about 0.07 nmol/mg protein in untreated cells to 0.8 nmoles/mg protein in cells treated for 14 days with nerve growth factor. Cells treated in suspension for 2-3 weeks with nerve growth factor do not express tetanus toxin binding sites; upon plating, these cells required one week for the appearance of binding sites, although neurites grew much more rapidly from these "primed" cells. The high binding capacity of these tetanus toxin sites as well as their sensitivity to neuraminidase, is indicative of a polysialoganglioside structure. We conclude that biotinylated tetanus toxin derivatives have many advantages, and that nerve growth factor-differentiated PC12 cells grown as monolayers are good models for the study of the development, localization, and function of neuraminidase-sensitive tetanus toxin binding sites.

E. Preservation of GABAergic Perikarya and Boutons After Transient Ischemia In the Gerbil Hippocampal CA1 Field

Occlusion of the carotid artery in the gerbil caused unilateral ischemia and is a good stroke model. Using an antibody directed against the  $\gamma$ -aminobutyric acid (GABA)-synthesizing enzyme glutamate decarboxylase (GAD) the fate of the GABAergic innervation was investigated in the hippocampal field CA1 of gerbils up to 14 days after a bilateral transient 5-min occlusion of carotid arteries. The CA1 pyramidal cells are subject to the ischemia-induced delayed neuronal death, first signs of which are detectable after 2 days, and which are fully developed after 4 days. Local GAD-immunoreactive neurons and boutons, however, exhibited no changes in their distribution and morphology over the whole 14-day period investigated, as studied both at the light and electron microscopic level. Thus, it can be assumed that the increased excitation observed during the development of delayed neuronal death, is not due

to a loss of GABAergic neuronal profiles. The resistance of the GABAergic neurons to the ischemic insult remains to be explained.

F. Monoamine Oxidase Inhibitory Activity of The Aminosteroid Iron Chelator, Lazaroid (U74500 A): Implications for Parkinson's Disease

The therapeutic value of monoamine oxidase (MAO) B inhibitors as adjuvants to L-dopa in the treatment of Parkinson's disease (PD) relies on potentiation of dopamine effect and reduction of oxygen free radicals, formed from  $H_2O_2$  generated by oxidation of dopamine. Such radicals can be generated as a result of interaction of  $H_2O_2$  with free tissue iron (Fenton Reaction), with ensuing membrane lipid peroxidation. The selective increase of free iron noted in the substantia nigra of Parkinson's brain has indicated a state of oxidative stress and increased lipid peroxidation, resulting from interaction of iron and with  $H_2O_2$  evolved from the two pathways of dopamine oxidation. Thus, the prevention of  $H_2O_2$  formation via inhibition of MAO and iron chelation would be an ideal therapeutic approach. Lazaroids (e.g. U74500A) are a group of noted 21-amino steroid iron chelators. They can penetrate blood brain barrier and act as potent membrane lipid peroxidative inhibitors. They are being investigated clinically for the treatment of trauma and stroke. Because certain iron chelators were known to inhibit MAO, we examined the action of lazarooids on the two forms of this enzyme in the rat brain and on iron induced lipid peroxidation. The 21-amino steroid ion chelators, 8-hydroxyquinoline, o-phenanthroline, 2,2'-dipyridyl and desferrioxamine showed a relatively greater selectivity for inhibition of rat brain MAO-A than MAO-B. The Lazaroid's inhibition of MAO was time-dependent and was not blocked by  $Fe^{2+}$  or  $Fe^{3+}$ , suggesting that MAO inhibitory and iron chelatory activity in such drugs are not mutually exclusive. These compounds were also effective in blocking iron induced lipid peroxidation and increased  $^{45}Ca^{+}$  uptake in intact rat cortical synaptosomes. Consideration should be given to this class of drugs as a means for retarding the neurodegenerative process of PD.

G. MPTP (1-Methyl-4-Phenyl 1,2,3,6-Tetrahydropyridine) Induced "Parkinsonism" in the Goldfish (*Carassius Auratus*)

The goldfish has been an important animal model for the study of processes involved in neurodevelopment, neurogeneration and neurodegeneration. We have extended this model to include studies concerning the neurotoxicity of MPTP on the possible dopamine (DA) neurons of the goldfish (*Carassius auratus*), and to correlate them with any behavioural or movement disturbances. We were further attracted to this system because the goldfish does not have a significant blood brain barrier, and our preliminary studies had shown the presence of monoamine oxidase (MAO) A and B activities, with relative  $K_m$  values of 180, 117, and 10  $\mu M$ , respectively, for tyramine, serotonin

and phenylethylamine deamination. The catecholamines in goldfish brain showed a substantial amount of noradrenaline and dopamine, whose concentration is uneven in the different brain regions. The highest content of DA and noradrenaline are found in the midbrain which also possesses the highest MAO and tyrosine hydroxylase activities. Intraperitoneal injection of MPTP (30 mg/kg) for 1 1/2 gm goldfish resulted in a profound reduction in movement, and a marked inability to swallow food. Time dependent (5 days) depletion of DA and NA, mainly from the midbrain region, was also observed. The effect of MPTP could be blocked by previous injection of the non-selective MAO inhibitor, tranylcypromine (10 mg/kg). The depletion of brain DA was specific since no change in retinal DA could be detected, even though the MPTP oxidative metabolite, MPP<sup>+</sup> was accumulated not only in the brain but also in the retina where MAO A and B are also present. The biochemical and behavioural changes induced by MPTP in the goldfish show parallelism to those reported in higher vertebrates. The data suggest that the goldfish may be a useful and viable model to examine the effects of MPTP and other neurotoxins on CNS.

#### H. Depolarization Induced ATP Release From Mouse Brain Synaptosomes: Calcium-Dependent and Independent Mechanisms

ATP is stored in secretory vesicles of presynaptic nerve endings. A sudden elevation of K<sup>+</sup> causes membrane depolarization and secretion of ATP together with specific neurotransmitters. We demonstrated here that ATP is secreted both in the presence of physiological external Ca<sup>2+</sup> concentration and in the absence of this divalent cation. The aim of our work has been to characterize the kinetics of both modes of ATP secretion from highly purified preparation of mouse brain synaptosomes. We measured the time course of ATP release using luciferin-luciferase included in the external solution. The rate constant of the ATP secretion increases with [Ca<sup>2+</sup>]<sub>o</sub>. In the absence of Ca<sup>2+</sup>, the release is slow. This calcium independent modality of ATP release could be induced by either KCl or RbCl but not by CsCl. These results are consistent with the idea of different permeabilities for K<sup>+</sup>, Rb<sup>+</sup>, and Cs<sup>+</sup> across K<sup>+</sup>-channels known to be present in the membrane of synaptosomes. Moreover, experiments conducted with impermeant anion isethionate keeping the product [K<sup>+</sup>]<sub>o</sub>X[Cl-1]<sub>i</sub> constant showed that ATP release was as in controls, indicating that ATP release was not due to osmotic lysis. Using potentiometric dye (DISC<sub>3</sub>(5)) we obtained an empirical relationship between [K<sup>+</sup>]<sub>o</sub> and membrane potential. The extent of ATP release follows a linear relationship with the membrane depolarization.

#### IV. ENDOTHELIAL CELLS IN ADRENAL MEDULLA AND BRAIN

##### A. Steroid Regulation of Monoamine Oxidase in the Chromaffin and Endothelial Cells of the Adrenal Medulla

Administration of different steroid hormones *in vivo* has distinct and specific effects on the MAO activity of the adrenal medulla. In an effort to reconstitute these effects in defined cells, we have isolated endothelial cells and chromaffin cells from the bovine adrenal medulla and tested each cell type for sensitivity to these steroids. As in the intact animal, we found that endothelial cell MAO activity was stimulated 1.5- to 2.5 fold by 10  $\mu$ M progesterone, hydrocortisone, and dexamethasone inhibited by ca. 50% by 17 estradiol, but unaffected by testosterone. The type of MAO in the endothelial cells was found to be exclusively of the A type. The chromaffin cells had MAO B exclusively and were inert to treatment with dexamethasone. The mode of action of the various steroids on MAO A activity in endothelial cells seemed to be that of affecting the number of MAO molecules, as binding of [ $^3$ H] pargyline, an MAO inhibitor, changed in proportion to changes in enzyme activity. Consistently, the kinetic parameters for MAO A showed changes in  $V_{\max}$  but not  $K_m$  under all conditions. The specificity of steroid action on MAO A activity was also supported by the fact that steroid induced changes in total cell division ([ $^{14}$ C]thymidine incorporation) and total protein synthesis ([ $^{14}$ C]leucine incorporation) were seen after changes in MAO A. We conclude that the differential effects of steroids on MAO activity in the intact adrenal medulla can be reproduced in cultured adrenal medullary endothelial cells but not in chromaffin cells. Therefore we suggest that the action of these steroid hormones on the intact adrenal medulla may be restricted to the endothelial cell component of this tissue.

##### B. Specific Adhesion Between Pheochromocytoma (PC12) and Adrenal Medullary Endothelial Cells in Co-Culture

Chromaffin cells in the adrenal medulla are found to close proximity to capillary endothelial cells, thereby forming the classical endocrine complex. To examine the possible chemical basis of their interaction in more detail, we have grown bovine adrenal medullary endothelial (BAME) cells in monolayer cultures and added to them pheochromocytoma (PC12) cells, a chromaffin tumor cell line of rats. The PC12 cells were chosen because of the similarities they share with adrenal medullary chromaffin cells. PC12 cells rapidly attached to BAME cell cultures, their rate of adhesion being significantly enhanced over binding of PC12 cells to either uncoated plates or to monolayers of unrelated cell cultures. Consistent with this observation, we noted that the extracellular matrix (ECM) derived from the BAME cells did not enhance PC12 cell adhesion and did not promote neurite sprouting as previously described for ECM derived from corneal endothelial cells. The specific adhesion between PC12 and BAME cells could be abolished by cell surface extracts derived from these two cells but

not by extracts derived from unrelated cell types. This activity was heat-labile, sensitive to trypsin and, to a lesser extent, to neuraminidase. We therefore conclude that PC12 cells may interact with BAME cells by specific proteinaceous adhesive factors associated with their plasma membranes. These interactions might represent the formative role of cell-cell contacts in the organization of the developing adrenal gland.

- C. Stimulation by IL-1 and Tumor Necrosis Factor (TNF-) or VonWillebrand Factor (VWF) Secretion By Rat Brain Microvascular Endothelial Cells (RBMEC) Plated on Matrigel, A Substrate Which Permits Sustained Expression of VWF

VWF synthesis and secretion is characteristic of endothelial cells (EC), but maintenance of expression of this molecule in a long term culture of RBMEC's has proved difficult. These difficulties are in marked contrast with results for EC cultures from large vessels, where physiologic properties have been more easily studied. Recently we found that RBMEC, when cultured on Matrigel®, develop the characteristic morphology of EC's and express VWF. We have quantitated the levels of VWF in the culture media using a sensitive ELISA method. We found that when RBMEC plated on Matrigel are compared to cells plated on uncoated dishes, only the cells plated on Matrigel® released detectable amounts of VWF in the culture media. Secreted VWF was detectable as soon as 5 minutes after introducing a fresh medium, reaching a maximum concentration after 6 hours. Stimulation of RBMEC with a mixture of IL-1 and TNF- resulted in a significant increase in secreted VWF. By contrast stimulation of RBMEC with lipopolysaccharide (100 g/ml) did not increase VWF levels in the culture media above the control. These data indicate that growth of RBMEC's on Matrigel in vitro permits expression and secretion of VWF in a manner similar to the behavior of these cells in situ.

- D. Hypertensive Rats Produce More Tumor Necrosis Factor Than Normotensive Rats in Response to Challenge with Lipopolysaccharide

Hypertension one of the most common risk factors for stroke. Hypertensive rats have been demonstrated to produce a high incidence of ischemic and hemorrhagic lesions in the brain stem, following a provocative dose of lipopolysaccharide (LPS). LPS stimulates macrophages to secrete the cytokines interleukin-1 (IL-1) and tumor necrosis factor- (TNF-), both of which convert the endothelium surface from an anticoagulant to a procoagulant state and thus may cause the production of focal ischemic lesions. In order to determine if increased TNF activity were one of the factors which may be responsible for the high incidence

of ischemic lesions in LPS-treated rats, we challenged spontaneous hypertensive rats (SHR) with 1.8 mg/kg LPS and measured TNF- levels in the blood and cerebral spinal fluid (CSF) of untreated SHR as well as LPS-challenged and unchallenged normotensive rates (WKY). We found that LPS induced a marked elevation of TNF-activity in both SHR and WKY. However, the response of the SHR was significantly higher than that found in the blood of WKY 2 h following challenge with LPS. Furthermore, when LPS was delivered intravenously, TNF activity was elevated mainly in the blood but not in the CSF. By contrast, when LPS was injected intracerebro-ventricularly, TNF- was particularly high in the CSF and much lower in the blood. These results strongly suggest that TNF- is produced locally in the brain and thus may cause a focal ischemic lesion. The higher incidence of stroke-like events occurring in SHR as compared to WKY may be related to the higher level of TNF- obtained after challenge with LPS.

#### V. Ascorbic Acid Detection, Metabolism and Function In Endocrine Cells and Leukocytes.

##### A. Ascorbic Acid within Chromaffin Granules: In situ Kinetics of Norepinephrine Biosynthesis

Ascorbic acid requirements for norepinephrine biosynthesis were investigated in intact bovine chromaffin granules using the physiologic substrate dopamine and a novel coulometric electrochemical detection high pressure liquid chromatography system for ascorbic acid. 10 mM external dopamine, 1 mM Mg-ATP, and 1 mM ascorbic acid was omitted, intragranular ascorbic acid was consumed in a 1:1 ration with respect to norepinephrine biosynthesis. The initial concentration of intragranular ascorbic acid was 10.5 mM, which was depleted in stepwise fashion to 15 lower concentrations over the range of 9.2 - 0.2 mM. Chromaffin granules containing these varying concentrations of intragranular ascorbic acid were then incubated with 1 mM exogenous ascorbic acid, and norepinephrine biosynthesis from dopamine was determined. The apparent  $K_m$  of norepinephrine biosynthesis for intragranular ascorbic acid was 0.57 mM by Eadie-Hofstee analysis and 0.68 mM by Lineweaver-Burk analysis. These data indicate that intragranular ascorbic acid is available and required for norepinephrine biosynthesis, that ascorbic acid is a true cosubstrate for dopamine  $\beta$ -monooxygenase, and that intragranular ascorbic acid is maintained by extragranular ascorbic acid. Continued norepinephrine biosynthesis in granules is dependent on both intragranular and extragranular concentrations of the vitamin. Furthermore, in situ kinetics of dopamine  $\beta$ -monooxygenase for ascorbic acid may be most accurately determined using intact granules and the true physiologic substrate.



**B. Ascorbic Acid Analysis Using High-Performance Liquid Chromatography with Coulometric Electrochemical Detection**

A method for the detection of ascorbic acid using high performance liquid chromatography with coulometric electrochemical detection and a technique for stabilization of the vitamin are described. Since less than 1 pmol of ascorbic acid can be detected, this assay provides significantly greater sensitivity than nearly all of the currently available procedures. Stabilization of 10 pmol or less of ascorbic acid at room temperature for up to 4 h and for several weeks at  $-70^{\circ}\text{C}$  facilitates storage of a large number of samples and measurement of ascorbic acid using an automated sampling device. This method was used to quantitate the amounts of ascorbic acid in human polymorphonuclear leukocytes and bovine adrenomedullary chromaffin granules. The calculated concentrations found for human neutrophils (1.35 mM) and bovine chromaffin granules (10.0 mM) are in agreement with previously published data. The assay is suitable for the determination of ascorbic acid in biological samples where only a small amount of tissue is available or very low amounts of ascorbic acid are found. This method is the first application of coulometric electrochemical detection to ascorbic acid HPLC analysis.

**C. Ascorbic Acid Transport and Accumulation in Human Neutrophils**

The transport, accumulation, and distribution of ascorbic acid were investigated in isolated human neutrophils utilizing a new ascorbic acid assay, which combined the techniques of high performance liquid chromatography and coulometric electrochemical detection. Freshly isolated human neutrophils contained 1.0–1.4 mM ascorbic acid, which was localized  $\geq 94\%$  to the cytosol, was not protein bound, and was present only as ascorbic acid and not as dehydroascorbic acid. Upon addition of ascorbic acid to the extracellular medium in physiologic amounts, ascorbic acid was accumulated in neutrophils in millimolar concentrations. Accumulation was mediated by a high affinity and a low affinity transporter; both transporters were responsible for maintenance of concentration gradients as large as 50-fold. The high affinity transporter had an apparent  $K_m$  of 2–5  $\mu\text{M}$  by Lineweaver-Burk and Eadie-Hofstee analyses, and the low affinity transporter had an apparent  $K_m$  of 6–7 mM by similar analyses. Each transporter was saturable and temperature dependent. In normal human blood the high affinity transporter should be saturated, whereas the low affinity transporter should be in its linear phase of uptake.

**D. Millimolar Concentrations of Ascorbic Acid in Purified Human Mononuclear Leukocytes**

Ascorbic acid (vitamin C) was found in isolated human mononuclear leukocytes and their purified components in millimolar concentration. Intracellular ascorbic acid was depleted  $>96\%$  during cell culture and was rapidly reaccumulated after addition of physiologic

56-kDa protein on S<sub>49</sub> plasma membranes. Labeling is dependent upon the interaction of the FluNCS-lectin with glycosylated receptor sites, since Nacetylgalactosamine, but not methyl mannoside, blocked labeling of the 56-kDa protein on S<sub>49</sub> membranes. In contrast, a random labeling pattern of membrane proteins was observed upon irradiation at 480 nm using other fluorescein conjugates, such as FluNCS-bovine serum albumin, (FluNCS-BSA) or FluNCS-soybean trypsin inhibitor (FluNCS-STI), which interact with cell membranes in a nonselective manner, or with N-(fluorescein-5-thiocarbamoyl)-n-undecyclamine (FluNCS-NHC<sub>11</sub>), which is freely miscible in the membrane lipid. Random labeling was also obtained by direct photoexcitation of [<sup>125</sup>I]INA at 314 nm, with no distinct labeling of the 88- and 56-kDa proteins in the respective membranes. These results suggest that protein ligands can be used to guide sensitizers to discrete receptor sites and lead to the selective labeling by photosensitized activation of [<sup>125</sup>I]INA. Site-directed labeling is obtained by an amplification process that locally and time-dependently intensifies the radioactive signal, thus revealing minor membranal components that could not otherwise be visualized by random labeling. This approach provides a method that offers new possibilities for application in different fields of chemical and biological research.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cytogenetics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P .I.: J.H. Tjio

Chief, Section on Cytogenetics, LCBG, NIDDK

## COOPERATING UNITS (if any)

UMD-New Jersey Medical School, Newark (E.S. Raveche), Univ. California, Berkeley (G. Brecher), Ernst Moritz Arendt Universitat, Greifswald, DDR (F. Herrmann), Humboldt Univ. Zoologisches Museum, Berlin (S. Santibanez).

## LAB/BRANCH

Laboratory of Cell Biology and Genetics

## SECTION

Cytogenetics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Sex reversal (studies with various treatments - hormones, temperature variation, population crowding by female or male sex) and assessment of possible spontaneous sex reversal on several fish spp. which had been reported previously by several authors is being continued. The cases reported on sex reversal in several coral fishes may be due to the fact that hermaphroditism is prevalent among coral fish spp. 2. Analysis of clonal hyperdiploid CD 5+B cells in mice with Dr. E. Raveche. Studies were designed to investigate the growth requirements for a transplantable line of hyperdiploid CD 5+B cells. The clonal CD 5+B cells are derived from NZB mice and may be responsible for the autoantibody production observed in these mice. The hyperdiploid CD 5+B cells are not only a model for such autoimmune diseases as rheumatoid arthritis and Sjogren's syndrome, they are very similar to the malignant cell in virtually all chronic lymphocytic leukemias. In all of these states, immunosuppression of normal B cell function is observed. Thus, a study of the hyperdiploid CD 5+B cells in NAB mice will help to dissect the growth requirements of CD 5+B cells in the human situation. 3. Kinetics of hemopoietic cell studies with Dr. G. Brecher. Enzymatic markers have replaced cytogenetic identification of sex matched donor cells. It seems that in addition to the T cell involvement, stromal incompatibilities between male and female syngeneic donors and hosts may be involved. 4. Post and prenatal diagnosis of karyotypic aberration studies and the genomic diagnosis of hemophilia A by R.F.L.P. analysis are being continued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  <b>Z01 DK 21019-08 LCBG</b>
PERIOD COVERED <b>October 1, 1989 through September 30, 1990</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Mechanism of hormone and transmitter secretion</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <b>P.I.: Harvey B. Pollard, Chief, Laboratory of Cell Biology and Genetics, NIDDK. Others: G. Lee, Ph.D., Res. Chemist; E. Rojas, Ph.D., VS; I. Abwater, Ph.D., RS; M. Levine, M.D., Sen. Inv., Med. Off.; A. Burns, Ph.D., Expert; M. Srivastava, Ph.D., FDA; C. McUtchen, Ph.D., Res. Phys.; G. Kuypers, Ph.D., VA; K. Magendzo-Weinberger, Ph.D., VF; A. Munoz, M.D., SV; V. Cera, Ph.D., M.D., SV; M. Li, M.D., VF; M. Shirvan, Ph.D., IRIA; A. Shirvan, Ph.D., IRIA; Y. Raviv, Ph.D., VF; M. Delafuente, Ph.D., VA; J. Fiedler, Ph.D., VA; C. Parra, Ph.D., VF; M. Kukuljan, M.D., VF; A. Goncalves, Ph.D., VA; A. Moura, Ph.D., VF; G. Goping, EM Tech.; P. Carroll, M.D., NSA, SV; D. Tombaccini, Ph.D., SV; P. Washko, DDS, SV; I. Cabentchik, Ph.D., SV; J. Heldman, SV; E. Heldman, Ph.D., SV; M. Yodanis, Ph.D., SV; D. Doron, M.D., SV; M. Abovero, Ph.D., IRIA; C. Cultraro, Res. Biol.; P. Caviedes, M.D., VA; F. Vargas, DDS, FDA, K. Ihariwal, Ph.D., VA; R. Welsh, Ph.D., SV</b>		
COOPERATING UNITS (if any) <b>Dipak Banerjee, Ph.D., University of Puerto Rico; Harry Haigler, Ph.D., University of California at Irvine; Jack Cohen, Georgetown University; John Hollenbeck, USUHS</b>		
LAB/BRANCH <b>Laboratory of Cell Biology and Genetics</b>		
SECTION <b>Cell Biology and Biochemistry</b>		
INSTITUTE AND LOCATION <b>NIH:NIDDK, Bethesda, Maryland 20892</b>		
TOTAL MAN-YEARS: <b>39</b>	PROFESSIONAL: <b>39</b>	OTHER: <b>0</b>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) <b>Our work continues to focus on the processes leading to fusion between granule and plasma membranes during exocytosis in cells, including chromaffin cells, beta cells from islet of Langerhans, nerve terminals, and mucin secreting human colon carcinoma cells. The contact and fusion event during exocytosis may be controlled by the protein synexin. We have recently cloned and sequenced the cDNA for this protein, and learned that it is a member of the annexin gene family. We have expressed synexin in E. coli and have found the recombinant protein to express both fusion and channel properties. Secretion mechanisms have also been studied in intact chromaffin cells, in which evidence has been found to support the concept that calcium and barium promote secretion by separate mechanisms. The acetylcholine receptor in chromaffin cells has been found to be different from the classical nicotinic receptor, inasmuch as it has unexpected muscarinic character. A novel calcium channel has been discovered in human beta cells, which responds to glucose and related metabolites. This channel is named by us the "G-type" calcium channel, and may play a central role in glucose activation of islets. Glucose induced insulin release is modulated by acetylcholine, and pharmacological analysis reveals that the muscarinic receptor type is M1 rather than M2. The adrenal medulla has provided a tractable system to study the interaction between endocrine cells and adherent capillaries. Endothelial cells have MAO-A, exclusively, and various steroids affect expression of this enzyme, without affecting the MAO-B which is exclusively found in chromaffin cells. A new method for measuring ascorbic acid using HPLC and coulometric electrochemical detection has been developed. The method allows measurement of the vitamin, not only in cells such as leucocytes and chromaffin cells, but also human plasma. Two transporters for ascorbic acid have been detected in human neutrophils. Millimolar concentrations of ascorbic acid have been detected in unbound form in both human neutrophils and</b>		

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY  
DISEASES

I. POLYAMINES

The aim of our work has been to elucidate the physiological function of the polyamines, and their role in growth and differentiation. For this purpose we have constructed a variety of mutants in both *Escherichia coli* and *Saccharomyces cerevisiae*, and have studied the phenotypic effects of the depletion of the intracellular polyamines. In addition, we have studied the enzymes involved in the biosynthesis of these amines, and their regulation *in vitro* and *in vivo*.

Our most recent studies on the phenotypic effects of polyamine deficiency in *E. coli* follow up our earlier observations that polyamine deficiency resulting from mutations in the genes coding for the biosynthetic enzymes causes the loss of the ability of the host cell to produce bacteriophage  $\lambda$  following either a lytic infection or lysogenic induction. The inability of  $\lambda$  to reproduce in these cells can be reversed by the addition of polyamines to the growth medium. These findings were in contrast to our results with wild type T4 or T7 bacteriophages since these phage would reproduce in the mutant cells even in the absence of added polyamines. We have now made polyamine-deficient mutants in a variety of other strains of *E. coli* and have shown that there are some strains of *E. coli* in which  $\lambda$  bacteriophage will reproduce even in the absence of endogenous or exogenous amines. A variety of  $\lambda$  strains (obtained from Drs. S. L. Adhya and S. Garges of LMB, NCI) were tested, and all showed the same effects. Hfr recombination studies have shown that another genetic defect is required in addition to the polyamine deficiency to produce the  $\lambda^-$  phenotype. We are continuing these studies in order to elucidate the role of polyamines in  $\lambda$  replication.

. . . . . Drs. C. W. Tabor and H. Tabor

Another phenotypic effect of polyamines that we have observed is the increased toxicity of paraquat, a known source of superoxide, in spermidine-deficient cells. This is of particular interest since this is the first striking effect specifically related to spermidine deficiency in *E. coli*; all of the other phenotypes of polyamine deficiency are not specific for spermidine and can be overcome by either putrescine or spermidine. Studies of the mechanism of this toxicity show that this is caused by superoxide formation and is associated with an increased binding of the paraquat (which is basic) to some polyanionic component(s) of the cell in the absence of polyamines. Studies with a glutathione-less mutant have shown that the increased paraquat toxicity in spermidine-deficient cells is not related to the absence of glutathionylspermidine. These findings are the basis of a convenient method that we developed for detecting the presence or absence of polyamine-producing plasmids.

. . . . . Drs. K. W. Minton (Uniformed Services University of the Health Sciences), H. Tabor, and C. W. Tabor

The above studies were carried out with mutants obtained by deletion of bacteriophage  $\mu$ . These deletion mutants grew in the absence of polyamines, albeit at a reduced rate. In our recent studies we have

used the techniques devised by Dr. C. Michael Cashel in an attempt to prepare mutants that have insertions as well as deletions in various parts of the *speEspeD* operon. Our current experiments indicate that such strains are not viable even if polyamines are added to the culture medium.

. . . . . Drs. Q.-W. Xie, H. Tabor, C. W. Tabor, and C. M. Cashel (LMG, NICHD)

We have continued our studies on the nature of the mutants that we have constructed in the biosynthetic pathway for polyamines in *S. cerevisiae*. By nucleotide sequencing we have identified the specific nucleotide change responsible for the "spe10" mutation, and have shown that this defect is in the *SPE1* gene. We have shown that this gene is on chromosome XI. Insertion-deletion mutants of the *SPE1* gene have been obtained, and have been shown to have an absolute requirement for amines for growth. Insertion-deletion mutants have also been obtained in *SPE2*, the gene for *S*-adenosylmethionine decarboxylase.

. . . . . Drs. Q.-W. Xie, D. Balasandarum, C. W. Tabor, and H. Tabor

We have determined the nucleotide sequence of the putative gene for ornithine decarboxylase from *Mycobacterium tuberculosis*.

. . . . . Dr. A. K. Tyagi

We have continued our studies on the structure and processing of the proenzyme form of *S*-adenosylmethionine decarboxylase. We have previously shown that this enzyme, both from *E. coli* and from *S. cerevisiae*, is first formed as a proenzyme, which is then processed post-translationally at a LysSer (*E. coli*) or GluSer (yeast) peptide to form two subunits, one of which contains an N-terminal pyruvoyl group derived from the serine moiety. The amino acid sequence derived from the nucleotide sequence of the yeast enzyme has been compared with derived sequence of the animal enzyme (Pegg et al.), and we have found that there is a striking conservation of seven of the eight amino acids immediately around the cleavage site, and 10 out of 12 amino acids located near the N-terminal portion of the proenzyme.

More extensive studies on the N-terminal amino acid sequence of the  $\alpha$  subunits of both the *E. coli* and the yeast enzyme before and after reductive amination, have shown that in addition to those subunits that have covalently-bound pyruvoyl groups, a substantial percentage of the subunits have an alanyl group in this position even before reductive amination. We have also continued site-specific mutagenesis studies on the effect of various mutations on the processing of the proenzyme. Preliminary results indicate that this processing reaction is a self-catalyzed reaction, i.e., that an exogenous cleavage enzyme is not involved.

. . . . . Drs. C. W. Tabor, H. Tabor, and D. T. Liu (DBB, CBER)

## II. YEAST RNA VIROLOGY

There are five families of double-stranded RNA (dsRNA) that replicate in cells of the yeast *S. cerevisiae*. Three of these, L-A, L-BC, and M, replicate in viral particles. A single-stranded RNA, called 20S RNA, also replicates in yeast.

We have developed *in vitro* template-dependent replication and transcription systems using opened empty L-A viral particles. We have used these systems to determine the sites necessary for these processes. The opened empty viral particles also specifically bind viral (+) strands, the RNA species that is packaged *in vivo* to form new particles. We have precisely defined this viral binding site and the structural requirements for the binding reaction. We have further proven that this binding site is the *in vivo* packaging signal for the L-A and M<sub>1</sub> viruses. Introduction of this packaging signal into a heterologous transcript results in the *in vivo* packaging of the heterologous RNA in L-A viral particles.

. . . . . Drs. T. Fujimura, R. Esteban, and R. B. Wickner

Our sequence of the entire L-A viral genome showed that it has two open reading frames (ORF), ORF1 occupying the 5' 40% of the L-A (+) strand and ORF2, overlapping ORF1 by 130 bp and comprising the 3' 60% of the (+) strand. ORF1 encodes the viral major coat protein (80 kDa) while ORF2 is expressed only as a fusion with ORF1 to form the 180 kDa minor coat protein. The C-terminal ORF2 domain has ssRNA-binding activity and its sequence includes a pattern diagnostic of RNA viral RNA-dependent RNA polymerases. This fusion protein is analogous to retroviral gag-pol fusion proteins.

We now have analyzed the mechanism by which ORF1 and ORF2 are fused to form the 180 kDa "gag-pol" fusion protein. We have shown that ribosomal frameshifting occurs at a site in the 130 bp overlap of ORF1 and ORF2. This site closely resembles the sites shown in HIV, RSV and other retroviruses to be responsible for the ribosomal frameshifting in those viruses including a "slippery" heptamer and a strong structure including an RNA pseudoknot. We have precisely defined the structural requirements for frameshifting in this system.

. . . . . Drs. J. D. Dinman and R. B. Wickner

We have constructed an L-A cDNA expression vector that can support M<sub>1</sub> replication in the absence of the L-A dsRNA virus. This requires both ORF1 and ORF2.

To determine the importance of L-A's conserved RNA polymerase sequence pattern, we have altered residues in this region. We find two domains essential for support of the M<sub>1</sub> satellite virus that include the conserved residues.

Expressing only ORF1 makes cells into ski<sup>-</sup> phenocopies, suggesting that one target of the anti-viral SKI products is the L-A major coat protein. We have recently cloned the SKI2 gene and its analysis may provide some information on this question.

. . . . . Drs. R. B. Wickner, J. C. Ribas, and W. R. Widner

The MAK3 chromosomal gene is necessary for replication of the L-A virus. We have cloned and sequenced this gene and find that it is most homologous to the *Escherichia coli* rim I gene encoding an N-acetyl transferase specific for the S18 ribosomal protein N-terminal alanine residue. We have found a consensus amino acid sequence pattern shared by N-acetyl transferases and MAK3 has this sequence pattern. Changing a conserved GF to SL eliminates MAK3 activity.

. . . . . Drs. J. C. T. Lopez and R. B. Wickner

We have shown that the 20S RNA of yeast, a species long known to be amplified when cells are placed in acetate medium, is a circular single-stranded RNA replicon. We have cloned and sequenced nearly the entire 3.0 kb genome and find that it encodes a protein of over 760 amino acids with sequence patterns characteristic of RNA-dependent RNA polymerases. The N-terminal region of the protein has sequence patterns typical of protein phosphorylation sites for the cAMP-dependent protein kinases.

. . . . . Drs. Y. Matsumoto, W. R. Widner, and R. B. Wickner

### III. NUCLEIC ACIDS

*Introduction.* L1 DNA (long interspersed repeated DNA, LINE 1 DNA) is a ubiquitous feature of mammals and comprises at least 10-20% of their genomes. L1 elements contain a promoter-like sequence at the left end, two highly conserved open reading frames (ORFs), and a guanine-rich polypurine:polypyrimidine sequence near the right terminus. Amplification of L1 elements has occurred repeatedly during mammalian evolution, and invasion by L1 elements into new sites is a frequent cause of polymorphism in mammals including humans. This can occur during the lifetime of the individual as was dramatically illustrated by a case of factor VIII deficiency due to L1 insertion in the factor VIII gene of a child whose parents both had the normal gene. We have been studying the L1 family of rats and describe below our recent findings.

*Current Findings.* We previously showed that the polypurine:polypyrimidine sequence at the right end of the rat L1 element adopts a series of abnormal DNA structures *in vitro* that may explain some of the properties of this end of the L1 element. These are: (1) its ability to induce strand uptake *in vitro* in a reaction that mimics early steps in the recombination process; and (2) its sensitivity to nucleases isolated from mammalian cells. We have suggested that these properties *in vitro* may explain the propensity of polypurine:polypyrimidine stretches to act as a hotspot for recombination *in vivo*.

We also showed that the L1 non-B structures compete with other types of non-B DNA structures for limiting amounts of supercoil energy. Therefore, the L1 polypurine sequence may affect a variety of other processes *in vivo* that are sensitive to the level of supercoil energy (e.g., gene expression or DNA replication).

Our current studies are aimed at extending the possible effects of the L1 polypurine:polypyrimidine region. We have shown that this region in RNA is a moderately strong arrest site for reverse transcriptase *in vitro*. This effect is probably related to stacking among purines analogous to what we have shown occurs on single-stranded and double-stranded templates with a variety of DNA-dependent DNA polymerases.



We also have demonstrated that the polypurine sequences dramatically reduce the activity of the L1 element promoter when situated upstream of it. This effect is orientation-dependent, and we are now examining the mechanism by which this occurs and also whether this effect is specific to the L1 promoter.

We are also examining the effect in mammalian cells of the possible genetic effects of the L1 polypurine:polypyrimidine region on contiguous DNA sequences. To do this we have introduced the L1 polypurine:polypyrimidine sequence in a shuttle vector designed by Dr. M. M. Seidman that permits the very sensitive assay in bacteria of genetic defects that occur in mammalian cells.

. . . . . Drs. K. Usdin and A. V. Furano

The L1 promoter-like region is a structurally complex 610 bp sequence that, as is the case with the corresponding region of the other mammalian L1 families, is species-specific. We are carrying out both transcriptional and deletion analysis of this DNA sequence. Although we have identified a major transcript that begins about 300 bp from the 5' end of the element we have recently found that the promoter-like region also greatly stimulates transcription from cryptic non-L1 promoters as far as 800 bp 5' from the 5' end of the L1 regulatory region. This transcript is by far the most prevalent one induced by L1, and preliminary analysis of it indicates that it terminates within the L1 regulatory region itself. We are now determining whether termination is due to a block in transcription or to an RNA processing event.

Deletion analysis indicates that the L1 regulatory region contains at least three different functional modules: The first 150 bp and last 250 bp are stimulatory modules that flank a central 200 bp inhibitory module. Accordingly the relationship between DNA concentration and promoter activity *in vivo* is complex since there is a competition for limiting amounts of both stimulatory and inhibitory transcriptional factors. We now have clones containing each of these modules alone or in different combinations and are analyzing their activity and transcriptional properties. We have identified a specific binding site in the first module for a putative stimulatory factor (see below).

. . . . . Drs. E. Pascale and A. V. Furano

The L1 promoter is unusual in that it does not contain binding sites for any of the general transcription factors. Using partially purified nuclear extracts, we have identified the location of the sites for sequence-specific DNA binding proteins within the promoter. The strongest site is a 15 bp region located in the first stimulatory module. This region contains a perfect version of a recently described factor binding site found in the regulatory region of the rat chymotrypsin gene. Rat acinar cells but not other cells contain a stimulatory transcriptional factor that binds to the site in the chymotrypsin gene.

. . . . . Drs. B. E. Hayward and A. V. Furano

In an attempt to assess the effect of L1 DNA on adjacent mammalian regulatory sequences, we examined as a model system the possible interactions between the L1 regulatory sequence and the promoter of the SV40

virus. To do this we examined the chloramphenicol acyl transferase (CAT) activity produced in cells transfected with various CAT fusion plasmids that contained the L1 sequence or the SV40 promoter/origin or various combinations of both. We found that there are at least two kinds of interactions. In one, the major regulatory elements of the SV40 promoter (i.e., the 21 bp repeats that bind the Sp1 general transcription factor, and the 72 bp enhancers) strongly stimulate the L1 regulatory sequence. The effect of the SV40 sequences is orientation-independent in monkey cells containing T antigen, and in these cells the interaction between the SV40 and L1 regulatory sequences increases the number of cells that can form active transcriptional complexes. By contrast the effect of the SV40 sequences is orientation-dependent in rat cells. In the second type of interaction we found that the L1 regulatory sequence can greatly stimulate replication from a repressed SV40 replicon. Since the L1 sequence is about 300 bp from SV40 origin, the effect of the L1 sequence is mediated by a mechanism different from that which is thought to explain the stimulatory effect of the SV40 regulatory elements on SV40 replication.

. . . . . Drs. E. Valle, E. Pascale, and A. V. Furano

To date neither of the putative proteins encoded by the two L1 open reading frames has been identified. To do this we have developed a series of cloning vectors to facilitate the isolation of functional L1 ORFs from genomic DNA. By using the polymerase chain reaction and primer oligonucleotides that are specific for ORF sequence containing either the initiation codon or the termination codon of either ORF we have amplified large quantities of ORF DNA from total rat genomic DNA. We are now cloning the ORF DNA in an expression vector so as to be under control of the bacteriophage T7 promoter. In this way we will be able to specifically synthesize in bacteria peptides of the size expected for either of the putative ORF-encoded proteins, and then screen them for biochemical activity using a variety of assays.

. . . . . Drs. B. E. Hayward, K. Usdin, and A. V. Furano

Screening for functional ORF-encoded proteins is complicated by the fact that most of the L1 elements in cells are defective. Computer analysis of the putative ORF II protein shows that it has motifs typical of reverse transcriptases. Therefore, we have devised a method to screen for reverse transcriptase in *E. coli*. The method is potentially very sensitive since only bacteria containing an active reverse transcriptase will survive the selection procedure. We are currently testing this method now which could be also used to screen for any reverse transcriptase.

. . . . . Drs. B. E. Hayward, K. Usdin, and A. V. Furano

The present day L1 families evolved independently from an ancestral L1 element that predated the mammalian radiation 80-100 million years ago. However, in spite of their length of time in the genome each present day family is rather new; i.e., most of the 50,000-100,000 members were generated within just the last few million years. To account for this apparent paradox it has been suggested that either extensive amplification of L1 did not occur in the past, or that old members are removed as new ones are generated.

However, we found that an ancestral rodent L1 family, which we call Lx, was extensively amplified about 10 million years ago and that about 60,000 copies of Lx are present in various modern murine genomes (Old World rats and mice). The occurrence of this amplification identifies those murine genera that shared a common ancestry and therefore defines the murine node in the lineage of modern muroid rodents.

Our results indicate that the relics of previous L1 amplifications could serve as excellent markers for determining the lineage of modern genera. In addition, the amplification of an L1 family would most likely greatly increase the genetic diversity of a population. This could predispose subsets of the population to genetic isolation which is a prerequisite for speciation. We plan to determine if the radiation of other rodent subfamilies is as closely related in time to the amplification of an L1 family as was the murine radiation.

. . . . Drs. E. Pascale, E. Valle, and A. V. Furano

We are studying the *E. coli* bacteriophage T4 as a model system for duplex DNA replication. Efficient DNA replication *in vitro* requires at least nine purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, the genes 41 and 61 proteins which function together as a primase and as a DNA unwinding enzyme (or helicase), RNase H, and DNA ligase.

*Crosslinking the T4 Replication Proteins to Specific Positions on the DNA Primer.* We have used DNA primers with photoactivated crosslinking reagents at specific bases to position individual components of the T4 multienzyme replication complex on the primer-template under active DNA synthesis conditions. The 5'-azido-2-nitrobenzoyl group was attached to thymidine by a 3-carbon tether to allow it to reach the major groove of DNA duplex. The primers used were two 34 mers of identical sequence, complementary to  $\phi$ X174 DNA, which differed in the position of the crosslinkable residue which was initially either 4 or 20 bases from the 3' primer terminus. The template sequence allowed DNA polymerase to elongate the primer by 5 residues with dTTP and dCTP, and 10 residues with dTTP, dCTP, and dGTP, thus moving the crosslinkable residue from 4 to 9 and 14, or from 20 to 25 and 30 bases behind the 3' primer terminus.

We find that polymerase is bound at the primer terminus and covers 5 to 7 bases of duplex DNA. Efficient crosslinking of the polymerase requires the genes 45 and 44/62 polymerase accessory proteins, gene 32 single-stranded DNA binding protein, and ATP. The 45 polymerase accessory protein is crosslinked 14 to 20 bases, and to a lesser extent 25 to 30, from the 3' primer end, but only in the presence of all 5 proteins and ATP. The 62 protein subunit of the 44/62 complex is most strongly crosslinked from 4 bases behind the 3' end, and crosslinking at this position is enhanced in the absence of polymerase. Crosslinking of 62 protein 20 bases behind the 3' OH is weaker and is increased in the absence of 45 protein or ATP. The 44 protein subunit of the 44/62 complex is not crosslinked in the presence of ATP. These studies position the 44/62 protein complex between polymerase and the 45 protein during ATP-dependent processive DNA synthesis.

We are currently analyzing the crosslinking in the absence of ATP and in the presence of the nonhydrolyzable analog ATP $\gamma$ S to determine which

steps in the assembly of the polymerase-accessory protein complex require ATP hydrolysis by the 44/62 protein ATPase. In the absence of ATP there is only weak crosslinking to 62 protein and polymerase. This crosslinking is not dependent on 45 protein, and 45 protein is not crosslinked at any position. ATP binding, assayed by using ATPγS, allows the formation of a complex of the three accessory proteins without polymerase. With ATPγS, there is tight binding of both components of the 44/62 protein complex with 44 protein crosslinked most strongly at 4 bases, and 62 protein at 14 bases behind the 3' end. Although 45 protein is only crosslinked weakly (and only at positions at least 20 bases behind the 3' end), 45 protein is required for 44 protein crosslinking and increases 62 protein crosslinking with ATPγS. Polymerase crosslinking does not increase above that found without ATP.

Formation of a complex which includes both polymerase and the accessory proteins requires ATP hydrolysis, and changes the crosslinking pattern of each protein. 45 protein is now crosslinkable, particularly at positions -14 and -20; 62 protein is now crosslinked to a lesser extent with the maximum at -4 rather than -14, 44 protein moves so that it can no longer be crosslinked to the primer at any position, and polymerase is strongly crosslinked at -4. ATP hydrolysis is sufficient to move 44 protein away from the primer, but both polymerase and ATP hydrolysis are necessary to crosslink 45 protein at -14 and -20. Since crosslinking of both polymerase and 45 protein to the primer in an ATP-dependent reaction also requires 44/62 protein, it is not clear whether polymerase and 45 protein interact directly or via their mutual interaction with the 44/62 complex which crosslinks between them on the primer strand.

. . . . . Dr. N. G. Nossal, in collaboration with Drs. T. Capson and S. Benkovic (Pennsylvania State University)

*T4 Phage Encodes an RNase H That Removes RNA Primers from the Lagging Strand.* We have previously purified an RNase H activity from T4 infected cells and shown that this enzyme can remove the pentamer primer (ACNNN) made by the T4 41/61 primase *in vitro*. The N-terminal amino acid sequence of a 33 kd polypeptide copurifying with this RNase H matches that of a predicted open reading frame of the same size in a region of the T4 genome upstream of the gene 33 transcription factor. We have now shown that this open reading frame encodes the RNase H by cloning it under the control of the T7 promoter in an expression vector. The protein expressed by this plasmid has the same size and RNase H and 5' to 3' DNA exonuclease activities as those of the enzyme from T4 infected cells. Biochemically the T4 RNase H most closely resembles the 5' to 3' exonuclease activities of *E. coli* DNA polymerase I and phage T7 exonuclease 6, which each hydrolyze both duplex DNA and the RNA strand of RNA-DNA duplexes. The T4 RNase H amino acid sequence has three regions with significant sequence similarity to those of the 5' to 3' exonuclease domains of *E. coli*, *Streptococcus pneumoniae*, and *Thermus aquaticus* DNA polymerase I's, as well as to the T7 exonuclease 6. We are currently using *in vitro* mutagenesis of an RNase H plasmid to construct an amber mutation which we will move into the T4 phage genome to test the function of the RNase H *in vivo*.

. . . . . Dr. N. G. Nossal and V. E. Chee

*Essential Regions of T4 DNA Polymerase.* T4 DNA polymerase, a single polypeptide of 103 kd, belongs to a family of replicative DNA polymerases which include human DNA polymerase α, yeast DNA polymerase I, and

the Herpes, Epstein Barr, and Vaccinia Virus DNA polymerases. Each of these polymerases contains six regions whose amino acid sequences are similar in each protein, separated by regions whose sequence and length differ between the polymerases. The similar regions presumably contain residues essential for polymerase activity, while the intervening regions may be involved in interactions between other replication proteins specific for each organism. As part of a study to define the functional domains of this family of polymerases, we have cloned the genes for T4 DNA polymerase from a temperature-sensitive antimutator DNA polymerase mutant *tsCB120* (L141), a second site intragenic allele-specific suppressor of *tsCB120* recently identified by Dr. Linda Reha-Krantz (University of Alberta), and the double mutant. We have purified the polymerase from each of these mutants and compared their activities alone, and as part of the T4 DNA replication complex. We find that even at the permissive temperature, the antimutator polymerase is defective in strand displacement leading strand synthesis from a forked DNA template in a reaction dependent on the three polymerase accessory proteins, 32 ssDNA binding protein, and the gene 41 DNA helicase. The polymerase with only the suppressor mutation catalyzes leading strand synthesis almost as well as the wild type, while the polymerase with both the antimutator and suppressor mutators has a rate of leading strand synthesis intermediate between the two single mutants.

. . . . . Drs. P. Spacciapoli and N. G. Nossal

*motA*-Dependent Expression of the Bacteriophage T4 Genes X.1, *uvvX*, 40, and 41. During infection, bacteriophage T4 transcriptionally regulates the expression of its genome, resulting in early, middle, and late products. Middle RNA arises both from the firing of new middle promoters and the elongation of upstream transcripts past transcription termination or processing sites. As a model for understanding how the phage regulates middle RNA, we are studying the expression of a cluster of T4 genes (5' → 3'): X.1 (newly identified DNA nuclease, see below), *uvvX* (recombination protein), 40 (stimulates head formation), and 41 (DNA replication protein, part of the primase-helicase).

Previously, we have shown that the two major 5' ends in the region from genes X.1 to 41 are located 225 bases upstream of X.1 and 190 bases upstream of *uvvX*. The regions upstream of each of these ends match the consensus sequence derived for T4 middle promoters dependent on the phage gene *motA*. We have demonstrated that these ends are indeed dependent on *motA* by S1 and primer extension analyses of RNA isolated from a *su<sup>+</sup>* or *su<sup>-</sup>* host after infection of the T4 *motA am* mutant, *amG1*.

In order to facilitate purifying *motA* protein, the *motA* gene has been cloned into a multicopy plasmid vector. The presence of the *motA* plasmid complements both T4 *amG1* and T4 *tsG1* (a temperature-sensitive *motA* mutant) for growth of the phage in a host that restricts T4 *motA* mutants (*E. coli* *tabG*). *MotA* protein has been expressed from a clone in which the *motA* gene is downstream of a T7 promoter. Fractions containing the expressed 25 kDa protein retard an oligomer containing the *motA*-dependent *uvvX* promoter sequence but not an oligomer containing an *E. coli* promoter during electrophoresis on polyacrylamide gels. This result indicates that the *motA* protein specifically binds to the *motA* promoter sequence.

. . . . . Dr. D. M. Hinton

**Identification of a New T4 DNA Nuclease.** Previously, we have identified an open reading frame designated X.1 immediately upstream of the *uvrX* gene in a genetically unmapped region of the T4 genome. We have cloned the X.1 gene downstream of the  $\lambda$  promoter  $P_L$  and partially purified the expressed 25 kDa protein. Fractions containing X.1 protein bind DNA and in the presence of  $Mg^{2+}$ , convert supercoiled plasmid to nicked circle and discrete, linear products. This cutting activity is stimulated by *uvrX* protein, suggesting that X.1 may be involved in a recombination/repair pathway with *uvrX*.

. . . . . Drs. M. Sharma and D. M. Hinton

#### IV. MEMBRANE STUDIES OF MACROPHAGES AND OF *ESCHERICHIA COLI*

**Aldoheptose Biosynthesis.** Previously, a novobiocin-hypersensitive mutant of *E. coli* K-12 carrying a *cysE-pyrE* linked mutation, designated *rfaD*, which specifically affects the synthesis of the aldoheptose, L-glycero-D-mannoheptose, has been isolated and genetically characterized. The *rfaD* gene codes for ADP-L-glycero-D-mannoheptose-6-epimerase, an enzyme required for lipopolysaccharide (LPS) core biosynthesis. The nucleotide ADP-D-glycero-D-mannoheptose accumulates in *rfaD* mutant strains. The *rfaD* phenotype includes increased permeability to a large number of hydrophobic drugs and dyes, and the formation of mucoid colonies. A 9-kilobase DNA *EcoRI* fragment carrying the *rfaD* gene was initially identified in the Clarke-Carbon Colony Bank cloned in pBR322, and subsequently smaller restriction fragments were cloned into several expression plasmid vectors. The proteins expressed by *RfaD*<sup>+</sup> plasmids, using several *in vivo* and *in vitro* expression systems, have been examined by SDS gel electrophoresis. *RfaD*<sup>+</sup> plasmids express a protein with a molecular weight of 37,000. Finally, ADP-L-glycero-D-mannoheptose-6-epimerase has been purified to homogeneity and partially characterized. The entire *rfaD* gene is located on a 1.3 kilobase *SspI-HpaI* fragment. This region and flanking regions have been completely sequenced, and the regulatory and coding regions have been determined.

. . . . . Drs. J. C. Pegues, L. Chen, A. W. Gordon, L. Ding, and W. G. Coleman, Jr.

#### V. ENZYME MECHANISM AND PROTEIN STRUCTURE

**Three-Dimensional Structure of the Tryptophan Synthase  $\alpha_2\beta_2$  Multienzyme Complex from *Salmonella typhimurium*.** We have continued to analyze the three-dimensional structure of the tryptophan synthase multienzyme complex which was previously determined by x-ray crystallography at 2.5 Å resolution. We are correlating the structural findings with information obtained on enzyme mechanism and on protein folding by other techniques. We are using the structure to select residues for further exploration by site-directed mutagenesis. X-ray crystallography of mutant and wild type forms of the enzyme in the presence and absence of ligands is in progress.

. . . . . Dr. E. W. Miles with Drs. C. C. Hyde and D. R. Davies (LMB, NIDDK)

**Spectrophotometric Studies of the Tryptophan Synthase  $\alpha_2\beta_2$  Complex from *S. typhimurium*.** We are comparing the chromophoric intermediates which are formed between pyridoxal phosphate and substrates at the active site

of the  $\beta$  subunit in the crystalline and soluble states. Polarized absorption microspectrophotometry is used to examine the formation of these intermediates in single crystals. These studies demonstrate that the enzyme is active in the crystalline state and provide information for x-ray crystallographic studies in the presence of ligands. We are also examining the effects of pH and of ligands on the equilibrium distribution of chromophoric intermediates. The results show that ligand-dependent site-site interactions occur both in solution and in the crystal.

. . . . . Dr. E. W. Miles with Drs. Andrea Mozzarelli, Alessio Peracchi, and Gian Luigi Rossi (University of Parma, Italy) and Peter Brzovic and Dr. M. F. Dunn (University of California, Riverside)

*Detection and Identification of Transient Intermediates in Reactions of Tryptophan Synthase.* We are using rapid-scanning stopped-flow spectroscopy to determine the rates of formation and decay of transient intermediates in the reactions of tryptophan synthase. We are investigating the effects of ligands on rates of reaction and the mechanism of channeling of indole by wild type and mutant forms of tryptophan synthase.

. . . . . Drs. E. W. Miles, Y. Sawa, and A. M. Kayastha with Peter Brzovic and Dr. M. F. Dunn (University of California, Riverside)

*Site-Directed Mutagenesis of the Tryptophan Synthase  $\alpha_2\beta_2$  Complex.* We are using recombinant DNA techniques to modify the *trpA* and *trpB* genes from *S. typhimurium*. Wild type and mutant forms of the  $\alpha_2\beta_2$  complex from *S. typhimurium* are expressed in very high yield and are crystallized from crude extracts. We are currently investigating the catalytic roles of several residues which are located in the pyridoxal phosphate binding site of the  $\beta$  subunit: glutamic acid residues 109 and 350, lysine 87, aspartic acid 305, and phenylalanine 306. We find that these residues have important roles in catalysis and in controlling reaction and substrate specificity.

. . . . . Drs. E. W. Miles, H. Kanzaki, Y. Sawa, and A. M. Kayastha

*Thermal Unfolding of the  $\alpha$  Subunit of Tryptophan Synthase.* Studies using scanning microcalorimetry have determined the denaturation temperatures and denaturation enthalpies for  $\alpha$  subunits from *E. coli*, *S. typhimurium*, and an interspecies hybrid. Circular dichroism studies have determined the effects of single amino acid substitutions at positions 49 and 60 on the thermal unfolding of the  $\alpha$  subunit from *S. typhimurium*. These studies identify residues in the  $\alpha$  subunit that affect stability.

. . . . . Drs. E. W. Miles and H. Kanzaki with Dr. Peter McPhie (LBM, NIDDK) and Dr. K. Yutani (Protein Institute, Osaka University, Japan)

*Channeling of an Indole Intermediate.* There is kinetic evidence that indole is a channeled intermediate in the coupled reactions catalyzed by the  $\alpha_2\beta_2$  complex of tryptophan synthase. The crystal structure of the  $\alpha_2\beta_2$  complex reveals the presence of a tunnel connecting the active

sites of the  $\alpha$  subunit and the  $\beta$  subunit. However, there has been no direct evidence for the existence of a channeled indole intermediate. We are now using rapid quench-flow studies under single-turnover conditions to demonstrate channeling. We are investigating the effects of mutation and of substrates on the time course of indole and tryptophan formation.

. . . . . Dr. E. W. Miles with Drs. Karen S. Anderson and Kenneth A. Johnson (Pennsylvania State University, University Park)

The interaction between fibrous rabbit muscle actin and four globular proteins in solutions of physiological ionic strength has been studied by means of sedimentation equilibrium and viscosity.

. . . . . Drs. S. Lakatos and A. P. Minton

Techniques for optimization of experimental protocols and data analysis in the quantitative characterization of macromolecular hetero-associations via measurement of sedimentation equilibrium have been carried out via simulation studies.

. . . . . Drs. S.-H. L. Hsu and A. P. Minton

The effect of high protein concentration upon the sedimentation coefficient of serum albumin has been carefully measured as a function of pH and ionic strength.

. . . . . Drs. S.-H. L. Hsu, S. Lakatos, A. P. Minton, and M. S. Lewis

Fully automated scanner control capability has been added to a custom-designed and built PC-based data acquisition system for the Beckman Model E analytical ultracentrifuge.

. . . . . Drs. A. P. Minton and M. S. Lewis

Glutathione is indicated to be a specific regulator of the activity of a valyl-tRNA synthetase, not replaceable by glutathionyl spermidine or cysteine. Since the initial functions of glutathione are still not defined, it is possible that this effect of glutathione might possibly represent a much more widespread and important regulatory system involved in protein biosynthesis, cell development, and neoplasia.

. . . . . Dr. S. Black

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems. Changes in calcium binding to fibrinogen upon clotting by thrombin were investigated and correlated with the release of fibrinopeptides and the polymerization steps.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and similarity of these sequences. The property of uniqueness (the occurrence of a *small* peptide at a frequency considerably less than that expected) has been



quantified, and speculations on this quantity and the immune response are under continued investigation.

. . . . . Drs. H. A. Saroff and E. Mihalyi

---

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 23,140-32 LBP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemistry of Sulfur-Containing Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Simon Black, Ph.D.

Chemist (Research)

LBP NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.0

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Glutathione is indicated to be a specific regulator of the activity of a valyl-tRNA synthetase, not replaceable by glutathionyl spermidine or cysteine. The protein complex in which the regulation occurs is suggested to be the "tip of an iceberg" representing a vast cytoplasmic apparatus that orchestrates protein biosynthesis and cell development. This system could be the seat of function of oncogene proteins that on mutation cause the regulatory malfunctions of cancer.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,330-12 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Aldoheptose Biosynthesis and Its Regulation and Hepatitis Non-A, Non-B

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	William G. Coleman, Jr. Ph.D.	Research Microbiologist	LBP NIDDK
Others:	Alfred W. Gordon, Ph.D.	IRTA	LBP NIDDK
	Lishi Chen, Ph.D.	Visiting Fellow	LBP NIDDK
	Li Ding, M.D.	Special Volunteer	LBP NIDDK
	Joyce C. Pegues, Ph.D.	Special Volunteer	LBP NIDDK

## COOPERATING UNITS (if any)

Belinda P. Seto, Ph.D., Division of Research Grants, NIH

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Pharmacology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.4

## PROFESSIONAL:

4.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

**Aldoheptose Biosynthesis.** An *E. coli* K-12 strain carrying a *cysE-pyrE* linked mutation, designated *rfaD*, which affects the synthesis of L-glycero-D-mannoheptose was isolated and genetically characterized. The *rfaD* phenotype includes, in addition to altered LPS synthesis, increased permeability to a large number of hydrophobic antibiotics. The *rfaD* gene initially identified in the Clarke-Carbon Genomic Bank was cloned in pBR322, and, subsequently, smaller restriction fragments were cloned into several plasmid vectors. The precise location of the *rfaD* gene on a 1.3-kilobase *SspI*-*HpaI* fragment has been determined. The *rfaD* gene and the flanking regions have been completely sequenced, and the coding and regulatory regions have been defined. The location of the *rfaD* gene on the physical map of the *Escherichia coli* chromosome has been resolved. *RfaD* plasmids express *in vivo* and *in vitro* a protein with the molecular weight of 37,000. The protein, ADP-L-glycero-D-mannoheptose-6-epimerase, has been purified to homogeneity and partially characterized. N-Terminal analysis of purified ADP-L-glycero-D-mannoheptose-6-epimerase confirms the first 34 amino acid sequence deduced from the nucleotide sequence of the *rfaD* gene coding region. The primary structure of the *rfaD* protein contains the sequence fingerprint for the ADP-binding  $\beta\alpha\beta$ -fold at the N-terminus.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,580-27 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mammalian Transposons

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Anthony V. Furano, M.D. Medical Officer (Research)  
and Chief, Section on Genomic Structure and Function, LBP LBP NIDDK

Others: Karen Usdin, Ph.D. Visiting Associate LBP NIDDK  
Bruce E. Hayward, Ph.D. Visiting Fellow LBP NIDDK  
Esterina Pascale, Ph.D. Visiting Fellow LBP NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Genomic Structure and Function

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.3

## PROFESSIONAL:

3.8

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Members of the L1 transposon family (long interspersed repeat DNA or LINE family) of rats are 6.7 kb long, 5 kb of which is devoted to protein-encoding sequences (ORFs). A very strong promoter is at the left end of the element, and a guanine-rich polypurine:polypyrimidine sequence is near the right end. Although the protein-encoding sequences of mammalian L1 families are highly conserved, the promoter sequences are completely distinct. This means that novel species-specific promoter sequences have been repeatedly acquired by L1 families during mammalian evolution and that these families have been amplified repeatedly in individual mammalian species. Our recent isolation of an L1 element that is ancestral to both the present day rat and mouse L1 families and is as highly repeated in both genomes support this scenario. Our previous demonstration that the rat L1 family contains a very active promoter sequence was the first evidence that L1 DNA is not just some non-functional "junk" DNA. We have further characterized this promoter and shown that some transcripts most likely begin about 300 bp from the 5' end of the sequence, that the 650 bp promoter consists of both inhibitory and stimulatory modules which can form numerous specific DNA:protein complexes with nuclear extracts as judged by gel retardation experiments. In addition, the L1 promoter exhibits a very strong (>20-fold) synergy with the SV40 promoter sequence and can strongly activate cryptic promoters as far as 800 bp distant from it. We previously showed that the guanine-rich polypurine:polypyrimidine tract at the right end of an L1 element destabilizes contiguous duplex DNA. We now find that this tract adopts several non-B DNA structures *in vitro* that may explain this phenomenon. We also found that three randomly chosen L1 insertion sites adopt abnormal DNA structures as well which might facilitate targeting of L1 elements to these regions. In addition the L1 and target site non-B structures compete for supercoil energy which *in vivo* might modulate the supercoil-dependent properties of L1 elements and their target sites.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 23,750-04 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Bacteriophage T4 Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Deborah M. Hinton, Ph.D.	Research Chemist	LBP NIDDK
Other:	Mridula Sharma, Ph.D.	Visiting Fellow	LBP NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Nucleic Acid Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.2

## PROFESSIONAL:

2.0

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

During infection, bacteriophage T4 transcriptionally regulates the expression of its genome, resulting in early, middle, and late products. Middle RNA arises both from the firing of new middle promoters and the elongation of upstream transcripts past transcription termination or processing sites. As a model for understanding how the phage regulates middle RNA, we are studying the expression of a cluster of T4 genes (5' to 3'): X.1 (newly identified DNA nuclease), *uvvX* (recombination protein), 40 (stimulates head formation), and 41 (DNA replication protein, part of the primase-helicase).

Previously, we have mapped major 5' ends of the X.1 to 41 RNA to 225 bases upstream of X.1 and 190 bases upstream of *uvvX*. The regions upstream of these ends match the consensus sequence derived for middle promoters dependent on the T4 phage factor *motA*. We have demonstrated that these ends are indeed dependent on *motA* by S1 and primer extension analyses of RNA isolated from a *su+* or *su-* host after infection of the T4 *motA am* mutant, *amG1*.

The *motA* gene has been cloned into a multicopy plasmid vector. The presence of the *motA* plasmid complements both T4 *amG1* and T4 *tsG1* (a temperature-sensitive *motA* mutant) for growth of the phage in a host that restricts T4 *motA* mutants (*E. coli* *tabG*). *MotA* protein has been expressed from a clone in which the *motA* gene is downstream of a T7 promoter. Fractions containing the expressed 25 kDa protein bind an oligomer containing the *motA*-dependent *uvvX* promoter sequence, retarding it during electrophoresis on polyacrylamide gels.

Previously, we have identified an open reading frame immediately upstream of the *uvvX* gene in a genetically unmapped region of the T4 genome. We have cloned the X.1 gene downstream of the  $\lambda$  promoter PL and partially purified the expressed 25 kDa protein. Fractions containing X.1 protein bind DNA and in the presence of Mg<sup>++</sup>, convert supercoiled plasmid to nicked circle and discrete, linear products. This cutting activity is stimulated by *uvvX* protein, suggesting that X.1 may be involved in a recombination/repair pathway with *uvvX*.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,140-24 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure and Function of the Tryptophan Synthase Multienzyme Complex. Was Tryptophan Synthase: Structure . . .

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Edith Wilson Miles, Ph.D.	Research Chemist	LBP NIDDK
Others:	Syed A. Ahmed, Ph.D.	Senior Staff Fellow	LBP NIDDK
	Arvind M. Kayastha, Ph.D.	Visiting Fellow	LBP NIDDK
	Xiang-Jiao Yang, Ph.D.	Visiting Fellow	LBP NIDDK

COOPERATING UNITS (# any) Drs.D.R.Davies and C.C.Hyde, LMB, NIDDK; P.McPhie, LBM, NIDDK; P.Brzovic and M.F.Dunn, Univ. of California, Riverside; A. Mozzarelli and G.L.Rossi, Univ. of Parma, Italy; K. Yutani, Osaka Univ., Japan; K.S.Anderson and K.A.Johnson, Pennsylvania State Univ., University Park, Pennsylvania

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Pharmacology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.7

## PROFESSIONAL:

2.5

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Our studies of the tryptophan synthase  $\alpha\beta\gamma$  complex are aimed at relating structure to function. The tryptophan synthase multienzyme complex is an excellent model system for investigating enzyme mechanism, protein-protein interaction, metabolite channeling, and ligand-dependent site-site interactions. We are continuing to correlate structural information obtained by x-ray crystallography of the tryptophan synthase  $\alpha\beta\gamma$  complex from *Salmonella typhimurium* with information obtained on enzyme mechanism and on protein folding by other techniques. We are also using the crystal structure to select residues for further exploration by site-directed mutagenesis. Our studies with mutants are clarifying the mechanism of the reactions catalyzed by the  $\beta$  subunit. We find that certain residues play catalytic roles whereas other residues control the reaction specificity or substrate specificity of the enzyme. Spectroscopic studies demonstrate that chromophoric intermediates are formed between pyridoxal phosphate and substrates at the active site of the  $\beta$  subunit both in solution and in the crystalline state. We find that the equilibrium distribution of intermediates is affected by pH, by ligands of the  $\alpha$  subunit, and by mutation. Rapid-scanning stopped-flow spectroscopic studies are used to measure the rates of formation of intermediates in the reactions of wild type and mutant forms of tryptophan synthase. The results shed light on the mechanisms of catalysis, of ligand-dependent site-site interactions, and of metabolite channeling. Scanning microcalorimetric and circular dichroism studies of the thermal unfolding of wild type and mutant forms of the  $\alpha$  subunit demonstrate features that are responsible for protein stability. Rapid quench-flow studies under single-turnover conditions provide evidence for the existence of a channeled indole intermediate.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,150-19 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Noncovalent Intermolecular Interactions in Biochemistry

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Allen P. Minton, Ph.D.	Research Chemist	LBP NIDDK
Others:	Susan Lakatos, Ph.D.	Visiting Associate	LBP NIDDK
	Shu-Hui L. Hsu, Ph.D.	IRTA Fellow	LBP NIDDK
	German Rivas, Ph.D.	Visiting Fellow	LBP NIDDK

## COOPERATING UNITS (if any)

M. S. Lewis, Biomedical Engineering and Instrumentation Branch, Division of Research Services, NIH; R. C. Chatelier, Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Pharmacology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.1

## PROFESSIONAL:

2.9

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The interaction between fibrous rabbit muscle actin and four globular proteins in solutions of physiological ionic strength has been studied by means of sedimentation equilibrium and viscosity.

Techniques for optimization of experimental protocols and data analysis in the quantitative characterization of macromolecular hetero-associations via measurement of sedimentation equilibrium have been carried out via simulation studies.

The effect of high protein concentration upon the sedimentation coefficient of serum albumin has been carefully measured as a function of pH and ionic strength.

Fully automated scanner control capability has been added to a custom-designed and built PC-based data acquisition system for the Beckman Model E analytical ultracentrifuge.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,260-24 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Nancy G. Nossal, Ph.D. Research Chemist  
and Chief, Section on Nucleic Acid Biochemistry. LBP LBP NIDDK

Others: Peter Spacciopoli, Ph.D. IRTA Fellow LBP NIDDK  
Vernon E. Chee, B. S. NIH Summer IRTA Fellow LBP NIDDK

## COOPERATING UNITS (if any)

Drs. Todd Capson and Stephen Benkovic, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Nucleic Acid Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 208

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.3

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are continuing our study of the *E. coli* bacteriophage T4 model system for duplex DNA replication in which efficient DNA replication *in vitro* is achieved with purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, the genes 41 and 61 primase-helicase, RNase H, and DNA ligase.

We have used DNA primers bearing photoactivated crosslinking residues to label 5 proteins of the T4 DNA replication complex: polymerase, the 44/62 and 45 polymerase accessory proteins, and 32 single-stranded DNA binding protein. We have determined the relative positions of each of the 5 proteins and the length of DNA in contact with the proteins. We find that the polymerase is bound at the primer terminus and covers 5-7 bases of duplex DNA. Efficient labeling of the polymerase requires all 5 proteins and ATP. When the crosslinkable residue is 14-20 bases from the 3' end of the primer-terminus, the 45 accessory protein is labeled, but only in the presence of all 5 proteins and ATP. When the primer-terminus is 25 bases from the crosslinkable residue, labeling of the 45 protein is greatly decreased. Our data suggest that the 44/62 complex resides between the polymerase and the 45 proteins.

*Identification of a T4 Gene Encoding the RNase H Which Removes RNA Primers on the Lagging Strand.* We have cloned the T4 DNA encoding an open reading frame which matches the N-terminal sequence of an RNase H which we previously purified from T4 infected cells. Plasmids containing this gene, located upstream of gene 33, express a protein whose size and RNase H and 5' to 3' DNA exonuclease activities are identical to those of the phage-encoded enzyme. This 35 kd protein has three regions with significant amino acid sequence similarity to those of the 5' to 3' exonuclease domain of *E. coli* pol I and the T7 gene 6 exonuclease.

*Essential Regions of T4 DNA Polymerase.* T4 DNA polymerase has six regions which share sequence similarity with eukaryotic DNA polymerases. To determine the function of shared and unique regions, we are characterizing altered T4 DNA polymerases produced by site-specific mutagenesis of the cloned gene.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,590-19 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Interactions of Biologically Important Macromolecules

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Harry A. Saroff, Ph.D. Research Chemist (Intermittent) LBP NIDDK

Other: Elemer Mihalyi, Ph.D., Ph.D. Special Volunteer LBP NIDDK

## COOPERATING UNITS (If any)

A. Patchornik, Weizmann Institute of Science, Rehovot, Israel; National Center for Drugs and Biologics

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Pharmacology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.6

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Cooperative binding systems are being studied taking into account site or sub-unit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems. Changes in calcium binding to fibrinogen upon clotting by thrombin were investigated and correlated with the release of fibrinopeptides and the polymerization steps.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and similarity of these sequences. The property of uniqueness (the occurrence of a small peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under continued investigation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,709-09 LBP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Polyamine Biosynthesis and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Celia White Tabor, M.D. Medical Officer (Research) LBP NIDDK

Others: Herbert Tabor, M.D. Supervisory Medical Officer (Research);  
Chief, Section on Pharmacology, LBP; and Chief, Laboratory of  
Biochemical Pharmacology LBP NIDDK  
Qiao-Wen Xie, Ph.D. Visiting Associate LBP NIDDK  
David Balasundaram, Ph.D. Visiting Fellow LBP NIDDK  
Anil K. Tyagi, Ph.D. Courtesy Associate LBP NIDDK

COOPERATING UNITS (if any)

D. T. Liu and N. Y. Nguyen, Division of Biochemistry and Biophysics, CBER; K. W. Minton, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.7

PROFESSIONAL:

4.2

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Polyamines (putrescine, spermidine, and spermine) are major cellular components, and have been shown to be involved in many systems related to growth and differentiation. Our current and older studies have been directed at learning how these polyamines are synthesized and regulated, and their physiological function. We have: (1) established the pathways for the biosynthesis of these amines in prokaryotes and eukaryotes and isolated the enzymes for the various steps in the pathways; (2) identified the genes responsible for each of the biosynthetic steps and constructed mutants with deletions in the various genes; (3) constructed plasmids that contain these genes and used the strains containing these plasmids to overproduce the encoded enzymes; (4) used the amine-deficient mutants to study the physiological effects of polyamine deprivation, including studies (in *E. coli*) on ribosome function,  $\lambda$  replication and paraquat toxicity, and (in yeast) on growth population and maintenance of the double-stranded RNA killer virus; (5) sequenced the gene coding for *S*-adenosylmethionine decarboxylase in both *E. coli* and *S. cerevisiae* and the gene coding for spermidine synthase in *E. coli*; (6) demonstrated that *S*-adenosylmethionine decarboxylase is first formed as a proenzyme in both *E. coli* and yeast and is cleaved post-translationally with the conversion of serine to a covalently-bound pyruvoyl group that is essential for activity; and (7) studied the effect of site-specific mutagenesis on the conversion of the proenzyme to the active enzyme.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,940-17 LBP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Yeast RNA Virology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Reed B. Wickner, M.D.	Medical Director, USPHS	
	and Chief, Section on Genetics of Simple Eukaryotes, LBP		LBP NIDDK
Others:	Tsutomu Fujimura, Ph.D.	Visiting Scientist	LBP NIDDK
	Jonathan D. Dinman, Ph.D.	IRTA Fellow	LBP NIDDK
	Yutaka Matsumoto, Ph.D.	IRTA Fellow	LBP NIDDK
	William R. Widner, Ph.D.	IRTA Fellow	LBP NIDDK
	Rosaura P.C. Valle, Ph.D.	Visiting Fellow	LBP NIDDK
	Hyun-Sook Lee, Ph.D.	Special Volunteer	LBP NIDDK
	Yang-Ja Lee, Ph.D.	Special Volunteer	LBP NIDDK
	Juan C. T. Lopez, Ph.D.	Special Volunteer	LBP NIDDK
	Juan Carlos Ribas, Ph.D.	Special Volunteer	LBP NIDDK

**COOPERATING UNITS:** Richard Fishel, Laboratory of Chromosome Biology, BRI--Basic Research Program, FCRF, Frederick, MD 21701; Rosa Esteban, Institute of Microbiology & Biochemistry, CSIS, University of Salamanca, Salamanca, Spain

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Genetics of Simple Eukaryotes

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

8.5

## PROFESSIONAL:

8.1

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The (+) strand of the L-A dsRNA virus of *Saccharomyces cerevisiae* has two large open reading frames, ORF1, encoding the major coat protein, and ORF2 that encodes a single-stranded RNA binding protein having a sequence diagnostic of viral RNA-dependent RNA polymerases. ORF2 is expressed only as a "gag-pol" type fusion protein with ORF1. We have constructed a plasmid expressing these proteins and show that it can support the replication of the killer toxin-encoding M1 satellite virus in the absence of an L-A dsRNA helper virus. ORF1 expression makes the strain a host *ski*-phenocopy, suppressing M1's requirement for MAK11, MAK18, and MAK27 genes, allowing a defective L-A (L-A-E) to support M1 replication and making a wild-type killer a superkiller. We have defined the packaging signal present on L-A (+) strands and on the (+) strands of satellite viruses of L-A. When this signal is inserted into foreign RNAs, those RNAs are packaged in L-A virus particles. We have demonstrated that ORF1 and ORF2 are fused to make the "gag-pol" fusion protein by -1 ribosomal frameshifting, the same mechanism used by retroviruses for this purpose. Our studies of this process in the case of L-A show that the strength of mRNA-tRNA binding at the ribosomal A-site is an important factor determining the efficiency of frameshifting. We propose that the fusion proteins of L-A and retroviruses use their pol domain to hold onto genomic RNA while their gag domain associates with gag molecules to prime coat formation.

ANNUAL REPORTS OF THE LABORATORY OF CHEMICAL BIOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Chemical Biology conducts research on the structure, function and dynamics of proteins and on molecular biology and genetics, especially as related to genetic diseases. A major emphasis of the Laboratory is in understanding the molecular processes involved in the developmental control of the expression of the human hemoglobin genes. A second major emphasis of the Laboratory is in the study of forces that stabilize globular proteins. A third program concentrates on cytogenetic analysis of patients with genetic illnesses. As part of the NIH Intramural AIDS Research Program, the Laboratory is working to characterize the tat protein of the HIV. Other new research initiatives include the cloning of the human erythropoietin receptor gene and the development of a transgenic mouse model of sickle cell disease. The protocol for treatment of sickle cell disease patients with hydroxyurea to elevate fetal hemoglobin has been enlarged to include protocols with the addition of recombinant human erythropoietin and also the treatment of patients with the thalassemia syndromes. A new program to establish a facility for the production of transgenic mice has been initiated.

The Laboratory has three Sections. The Section of Protein Chemistry and Conformation, under Dr. Hiroshi Taniuchi, is devoted primarily to studying the folding and assembly of globular proteins, especially cytochrome c. The Section on Molecular Forces and Assembly is the home of the Cytogenetics Unit under Dr. Beverly White, which is a joint endeavor of the Inter-Institute Genetics Program of the Clinical Center and NIDDK. The Section on Molecular Biology and Genetics, under Dr. Alan N. Schechter, is concerned primarily with the molecular basis of the developmental control of gene expression, especially in human erythroid cells, and its relevance to the understanding of the molecular basis of disease states and possible approaches to their therapy.

During the last year several personnel changes have occurred. Dr. Griffin Rodgers, a Senior Staff Fellow, has been approved for a tenured position in this Laboratory. Dr. Betty Peters has been awarded a Robert Wood Johnson Foundation Minority Medical Faculty Development four-year fellowship. Dr. Nobuhiro Uyesaka of Nippon Medical School has been in residence under the Courtesy Associate Program for senior scientific visitors. Dr. C. B. Anfinsen continues as a Scientist Emeritus in this Laboratory and is in frequent contact with the activities here.

Extensive research collaborations exist within this Laboratory and with other Laboratories in this Institute, in NIH, and nationally and internationally as outlined in the individual Research Project Reports. A formal collaboration has been established with the Clinical Center's Inter-Institute Medical Genetics Program to fund a clinical and research cytogenetic program. Clinical collaborations also exist with the Clinical Hematology Branch of NHLBI and other units. It is under the aegis of this collaboration that the clinical studies of hydroxyurea treatment of sickle cell patients is done. Development of transgenic sickle cell mice is being done in collaboration with the Metabolic and Developmental Neurology Branch of NINCDS. In addition, a formal collaboration has been established involving the exchange of personnel and resources with Dr. David Hankins of the Laboratory of Experimental Hematology of the Armed Forces Radiobiological Research Institute at the National Naval Medical Center (who is now transferring to the faculty of

George Mason University in Virginia) and the Division of Hematology of the Children's National Medical Center. The participation of this Laboratory in the NIH Inter-Institute Medical Genetics Program and the NIH-George Washington University Hematology Training Program continues to grow. The Laboratory is now also a major part of the recently established Intramural AIDS Research Program. The work in this program involves collaborations with KabiGen AB of Stockholm, Sweden. Other collaborations include those with the Department of Pediatrics of Johns Hopkins Medical School and the Department of Medicine at the University of Mississippi School of Medicine; the INSERM Unit 15 in Paris, France; the Hasharon Hospital in Israel; and the Medical Genetics Institute of the Children's Hospital of Shanghai, China.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25008-27 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**The Core Loop Interaction That Controls Protein Folding**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

**PI:** **Hiroshi Taniuchi** **Chief, Section on Protein** **LCB, NIDDK**  
**Chemistry and Confirmation**

**Others:** **Alice Fisher** **Chemist** **LCB, NIDDK**  
**Greg Charles** **Biological Aid** **LCB, NIDDK**

## COOPERATING UNITS (if any)

University of Padova, Padova, Italy

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Protein Chemistry and Conformation

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.55

## PROFESSIONAL:

1.0

## OTHER:

0.55

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanism by which the assembled polypeptide chain folds to the three-dimensional structure is a subject of intensive studies in many laboratories. We have taken an approach to cracking this second half of the genetic code by investigating a previously unknown non-covalent interaction. Our studies in the previous years have suggested that such new interaction comes to existence in the form of core interaction loop after folding. As described in the previous years we have prepared Type 1, 2, 4 and 5 fragment complexes from horse, tuna, yeast iso-1 or Candida cytochrome c. The different types represent the different sites of the discontinuity of the polypeptide chain. We have shown that the heme and apofragments of Type 1, 2, 4 or 5 complexes are completely exchangeable between any two of the above 4 species. Furthermore, with the exception for two Type II hybrid complexes, all homologous and hybrid Type 1, 2 or 5 complexes exhibit the 695 nm absorbance band. Y. P. Myer and colleagues have shown that the 695 nm band, indicative of the Met 80-S-heme Fe bond, monitors the integrity of the Trp 59-heme domain. Our results show that the 695 nm band also monitors the structural integrity of the regions above and to the right of heme. Monoclonal 4-128-6 which recognizes the structure above the heme of yeast iso-1-cytochrome c (see the previous reports) was found to cross react with all Type 1 and 2 hybrid complexes containing yeast iso-1-apofragment or apoprotein. Monoclonal 4-74-6, recognizing the structure below and to the left of the heme (see the previous reports) was also found to cross react with all Type 1, but not Type 2, hybrid complexes containing yeast iso-1-apocytochrome c. Combination of two or more mutations occurs in the right channel of the hybrid complexes. The right channel found by R. E. Dickerson and colleagues is formed by close contact of the COOH-terminal helix with the NH<sub>2</sub>-terminal helix and the heme. Thus, the results suggest that all residues of the right channel are likely exchangeable between any combination of two of eukaryotic cytochrome c species. This, in turn, suggests that the core interaction loop of the right channel is conserved. Thus, the present studies point to the fundamental importance of the core interaction loop in protein folding.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25011-16 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**The Core Interaction Loops and Core Loop Coalescence Energy in Protein Folding**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: **Hiroshi Taniuchi**Chief, Section on Protein  
Chemistry and Conformation

LCB, NIDDK

Others: **Alice Fisher**

Chemist

LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

**Laboratory of Chemical Biology**

## SECTION

**Section on Protein Chemistry and Conformation**

## INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

## TOTAL MAN-YEARS:

**0.8**

## PROFESSIONAL:

**0.8**

## OTHER:

**0**

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

As described in the previous years, our studies of protein folding have led to the hypothesis that (1) a previously unknown non-covalent interaction exists in the hydrophobic cores of proteins; (2) this new interaction is exothermic and mediated by the contacting groups which form a closed loop (core interaction loop) in the core; and (3) the core loop interaction is sensitive to the detail of the group contact and therefore has the ability to order the core groups.

To know more about the nature of this core loop interaction, we have measured  $K_D$  for the complete set of homologous and hybrid complexes of type 1, 2, 4 and 5 (64 in total number) prepared from horse, tuna, yeast iso-1 and Candida cytochromes c and the mid point of thermal transition of the 695 nm absorption band for selected complexes. The different types represent the different discontinuity sites of the polypeptide chain. The value for  $K_D$  was found to vary depending on which species of heme or apoprotein (or apoprotein) was used or which combination of heme and apoproteins (or apoprotein) was used. Analysis of the data and comparison of the amino acid sequences have allowed us to assign 6 mutations responsible for  $K_D$  changes. Furthermore, the interaction energy affected by such mutations is non-additive. Such non-linear behavior of the interaction is expected for the core loop interaction. Of the 6 mutations affecting  $K_D$ , 4 were found to be located in the core of the right channel (see another report). In the previous years we have assigned 3 more folding units to cytochrome c in addition to the right channel. We assume that each of these folding units are associated with a core loop.

Furthermore, the present evidence, combined with the studies in the previous years, suggest that the 4 core loops are coalesced in the ground state and segregated at the activated. The present results have allowed us to calculate the core loop coalescence energy separately from the conventional conformational energy. The calculated core loop coalescence energy (-5.25 kcal/mol at 24 degree C) of horse cytochrome c accounts to approximately 72 per cent of folding energy. Furthermore, the calculated perturbation of the core loop coalescence energy (-4.68 kcal/mol at 24 degree C) by Ile 9 to Leu and Leu 64 to Met mutations essentially explains the difference of folding energy between horse and yeast iso-1-cytochrome c found by E. H. Zuniga and B. T. Nall.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25016-17 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-Acting Factor(s) Controlling Globin Gene Expression in K562 Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alan N. Schechter

Chief

LCB, NIDDK

Others: Moshe Mittelman  
Harish DaveVisiting Associate  
Guest WorkerLCB, NIDDK  
LCB, NIDDKCOOPERATING UNITS (if any) Department of Pathology, USUHS, Bethesda, MD (Dr. Pablo Gutman);  
Connaught Laboratories, Toronto, Canada (Dr. S. X. Cao)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐
- (a) Human subjects
- 
- ☐
- (a1) Minors
- 
- ☐
- (a2) Interviews

☒ (b) Human tissues☐ (c) Neither

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

K562 is an erythroleukemic cell line used as a model for the study of the control of human globin gene expression. These cells do not support transcription of the beta-globin gene but do express transcripts of epsilon- and gamma-globin genes at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence of trans-acting factors which exert their action on sequences corresponding to the promoters of these genes. Sequence specific DNA binding protein acting on cis-regulatory elements have been hypothesized to be key elements in eukaryotic gene transcription, and even though considerable progress has been made in their isolation, only one DNA binding proteins with affinity for the human globin gene promoters have been identified. We have defined several positive and negative regulatory regions 5' to the epsilon-globin promoter, and detected binding of proteins to these regions.

We have previously reported the presence of a transcriptional control element with properties of a silencer extending from -392 to -177 bp relative to the cap site of the human epsilon-globin gene. We also showed that this silencer has stronger inhibitory activity in HeLa cells than K562 human erythroleukemia cells. Using deletion mutants and synthetic oligonucleotides in transient expression assays, DNA sequences responsible for this effect have been further delimited to 44 nucleotides located between -294 and -251 bp. Gel electrophoresis mobility shift assays and DNaseI footprinting assays demonstrate that these negative regulatory sequences are recognized differently by proteins present in nuclear extracts obtained from HeLa and K562 cells. The protein present in K562 cells, but not in HeLa cells, that interacts specifically with this silencer binds to the same sequence recognized by the yeast silencer binding protein ABF1. Possible mechanisms by which these proteins may regulate epsilon-globin gene transcription in erythroid and non-erythroid cells are discussed.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25021-15 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sickle Cell Anemia: The Intracellular Polymerization of Hemoglobin S

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Constance Tom Noguchi Research Physicist LCB, NIDDK

Others: Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any) LCDB, NIDDK (J. Blanchette-Mackie); NINCDS (Dr. S. Karlsson), Birmingham, U.K. (Dr. J. Stuart), Johns Hopkins (Dr. S. Charache), University of Calif., San Francisco (Dr. N. Mohandas), Sydney Hospital, Australia (Dr. E. Raik).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hemoglobin S polymerization is determined by the oxygen saturation, hemoglobin concentration and hemoglobin composition. Within the erythrocyte, hemoglobin polymerization can lead to abnormal rheology. Evaluation of the polymerization potential of a whole cell population has been difficult due to heterogeneity in hemoglobin concentration and hemoglobin composition. These heterogeneities are reflected in the broad density distribution of sickle erythrocytes and the variable distribution of fetal hemoglobin. We are now working on an analysis of sickle hemoglobin polymerization that would enable us to explicitly consider cell heterogeneity in hemoglobin concentration, composition, and possibly even oxygen affinity. Such an analysis would be useful not only in evaluation of individual sickle cell patients, but also in assessment of therapeutic strategies which alter hemoglobin concentration or hemoglobin composition, and in the evaluation of the transgenic mouse model for sickle cell disease with mixtures of sickle hemoglobin and mouse hemoglobins.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25025-14 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Origin of Specificity of Antigen-Antibody Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi Chief, Section on Protein Chemistry and Conformation LCB, NIDDK

Others: Antonello Punturieri Visiting Fellow LCB, NIDDK  
Paola Rizzo Visiting Fellow LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Protein Chemistry and Conformation

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.67

## PROFESSIONAL:

1.67

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

As described in another report, the studies of protein folding in this Section have led to the hypothesis that (1) a previously unknown non-covalent interaction exists in the hydrophobic cores of proteins; (2) this new interaction, called the core loop interaction, is exothermic and mediated by the contacting groups which form a closed loop in the core; and (3) the core loop interaction is sensitive to the detail of the group contact and therefore has the ability to order the core groups.

Antigen-antibody interaction resembles this core loop interaction in two critical aspects. First, both the core loop interaction and antigen-antibody interaction recognize a single amino acid substitution even if no polarity - change is involved. Second, both the core loop and the interface between antigen and antibody are devoid of solvent. Thus, we speculate that the specificity of antigen-antibody interaction has its origin in the core interaction loop which would form within or across the antigen antibody interface. To test this idea 6 hybridoma cell lines producing IgG monoclonals to yeast iso-1-cytochrome c have been prepared in the previous years.

Our strategy is that (1) cloning the cDNA of the individual monoclonals; (2) sequencing of the cDNA to deduce the amino acid sequences; (3) constructing the three dimensional structures of monoclonals by computer homology modelling to examine the antigen binding sites; (4) developing an expression system for the cloned cDNA; (5) identifying the hydrophobic cores of the domains of the monoclonal molecules by computer modelling; (6) mutating the core residues (one at a time) by site directed mutagenesis; (7) expressing the mutated cDNA to examine the influence of the mutation on antigen-antibody interaction; and (8) if the results of (7) are positive, (7) will be repeated with different core residues of the same domain and also with the core residues of different domains to map the groups which influence antigen-antibody interaction. If the hypothesis is correct, the core groups remote from the antigen binding site should influence the antigen-antibody interaction.

In the current year cloning cDNA prepared from mRNA of hybridoma cell line 2-96-12 (one of the above 6 hybridoma cell lines) has been carried out. The cDNA library (phages) thus obtained was screened for the presence of clones containing the kappa light chain cDNA. Thus, 37 positive clones were purified.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25028-12 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**The Development of Non-Invasive Methods to Assess Sickle Cell Patients**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Griffin P. Rodgers Senior Investigator LCB, NIDDK

Others: Constance T. Noguchi Research Physicist LCB, NIDDK  
Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (if any)

Clinical Hematology Branch, NHLBI (A.W. Nienhuis); Clinical Branch, NEI (M. Roy);  
Transfusion Medicine, CC (H. Klein); BEIB (Eli Walker); Biometry Branch, NEI  
(M. Podgor); MRC Laboratory, Kingston, Jamaica (G. Serjeant).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The molecular and cellular pathophysiology of the sickle cell syndromes are now appreciated with a great deal of precision. On the other hand, our understanding of the relationship of these subcellular events to the variable clinical expression of sickle cell disease remains largely speculative. A major effort of our research group has been to develop quantitative ways to clarify disease pathogenesis, as well as to assess severity and progression. Using calibrated phthalate ester separation method, which we previously described, we have now defined at least three cellular processes contributing to the extensive red cell heterogeneity that is commonly observed in the sickle cell syndromes. Using a sensitive nickel mesh filtration system, we are systematically determining the effects of changes in oxygen saturation, corpuscular hemoglobin concentration/compition and temperature of the flow properties of sickle hemoglobin containing erythrocytes. Ophthalmological studies of the patients show highly significant correlations between the extend of erythrocyte heterogeneity with conjunctival and retinal vessel pathology. As predicted by biophysical studies of polymer formation, we find that treatment of steady state sickle cell patients with selective arteriolar vasodilators results in a significant resolution of both acute conjunctival and retinal abnormalities, as well as an improvement in color vision performance. These beneficial effects occurred in the absence of a direct drug-induced inhibition of polymer formation, and therefore suggests that inappropriate vasospasm or frank vasoconstriction, perhaps in response to the altered rheology of red cell containing polymerized sickle hemoglobin is a significant contributing factor to the pathogenesis of sickle cell disease. This conclusion is also supported by our recent observation that "relative" hypertension is a significant risk factor for the occurrence of stroke in sickle cell patients. Using the technique of laser-Doppler velocimetry, and phlethysmography we have found that forearm cutaneous microcirculatory flow is sickle cell individuals show characteristic patterns, which may become more "normalized" depending upon the fraction of non-S hemoglobins and during crisis. We hope that these cellular and physiological measurements will allow us to understand better the extreme spectrum of disease manifestations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25038-10 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of HTLV-I Tat-I Product on Globin Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Henry B. Fox Staff Fellow LCB, NIDDK

Others: Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (if any)

Armed Forces Radiobiological Research Institute, Bethesda, MD  
(Drs. W. D. Hankins, and H. F. Fox)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25045-07 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Beta Globin Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Patricia Berg	Senior Staff Fellow	LCB, NIDDK
Others:	Robert H. Broyles	Guest Worker	LCB, NIDDK
	Shu-zhen Huang	Visiting Associate	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any) Shanghai Institute of Medical Genetics, Shanghai Children's Hospital, Shanghai, China (Dr. Y.-t. Zeng); Shanghai Institute of Cell Biology, Shanghai, China (Dr. R.-L. Qian); Hopital Robert Debre, Paris, France (Dr. J. Elion); Institut National de la Sante et de la Recherche Medicale Unit 15,

LAB/BRANCH Paris, France (Dr. D. Labie)

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Regulation of the beta globin gene is important for better understanding hemoglobinopathies such as sickle cell anemia and beta thalassemia, yet this regulation is poorly understood. We are currently studying two regions of DNA which appear to be important in this respect, one extending from the cap site upstream to -600 bp and the other the second intervening sequence (IVS2).

The area upstream of the beta globin gene can be subdivided into several regions. The first 100 bp, or proximal promoter, is known to contain sequences important for both the correct initiation of transcription and for the amount of mRNA synthesized. The next 100 bp, from -100 to -200, is called the distal promoter because it seems to contain sequences required for erythroid specific transcription of the gene. We have found, by deletion analysis, that DNA between -233 and -185 is necessary for gene expression. In addition, we have defined two regions that act as silencers or negative regulatory elements, one between -610 and -490 bp, the other between -338 and -233 bp. We have also identified a protein (BP1) which binds to both silencers, making it a candidate for a repressor protein. Consistent with this possibility, BP1 binds more tightly to the DNA of sickle cell anemia patients with the Indian haplotype who produce less beta protein than normal and are relatively healthy. Conversely, BP1 binds more weakly to the DNA from patients with the sickle cell Bantu haplotype, who exhibit much more severe clinical symptoms. Further studies are underway examining other haplotypes.

In vitro experiments have shown that IVS2 is required for expression of the beta globin gene, although its function is not known. There is also a class of thalassemic patients who have a single base mutation in IVS2 and who make no beta protein. We have identified binding sites for at least two known proteins in this region, NFI (nuclear factor I) and eryf1 (or GF1), an erythroid specific protein. Using purified factors, we see much stronger binding of eryf1 to this region in the normal DNA than the mutant. eryf1 is known to be a positive acting transcription factor, so decreased binding might cause decreased transcription. Our data suggest that NFI and eryf1 may be interacting to cause this effect.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25056-06 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Human T Cell Receptor Delta and Alpha Gene Usage

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Jean-Pierre de Villartay Visiting Fellow LCB, NIDDK

Others: David Cohen Medical Officer LCB, NIDDK

## COOPERATING UNITS (if any)

Washington University School of Medicine (Dr. S. Korsmeyer); Lab. of Tumor Cell Biology, NCI (Dr. E. Tschachler) Metabolism Branch, NCI (Drs. C. Glenn Begley, M. P. Davey, T. A. Waldmann), Navy Medical Oncology (Dr. Ilan R. Kirsch)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25057-06 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Function, Ligand, and Ontogeny of Expression of the Gamma/Delta T Cell Receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Amy B. Pullman

Guest Researcher

LCB, NIDDK

Others: David I. Cohen  
J. P. de Villartay  
Lisa JacobsMedical Officer  
Visiting Fellow  
BiologistLCB, NIDDK  
LCB, NIDDK  
LCB, NIDDK

## COOPERATING UNITS (if any)

NIAID, NIH (Drs. J. Coligan and E. Shevach); NIC, NIH (Dr. Jeffrey Cossman);  
FDA (Dr. L. Matis)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

PROFESSIONAL

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐
- (a) Human subjects
- 
- ☐
- (a1) Minors
- 
- ☐
- (a2) Interviews

☒ (b) Human tissues☐ (c) Neither

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25058-05 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Laboratory Model of Adult Globin Gene Expression**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Griffin P. Rodgers	Senior Investigator	LCB, NIDDK
Others:	Houria Debabeche	Guest Worker	LCB, NIDDK
	Patricia Berg	Senior Staff Fellow	LCB, NIDDK
	Alan N. Schecher	Chief	LCB, NIDDK

## COOPERATING UNITS (if any)

MRC Unit, University of West Indies, Kingston, Jamaica (Dr. G. Serjeant).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the molecular mechanisms which control the individual and total concentrations of hemoglobins in human erythrocytes. The study of the control of hemoglobin levels has direct relevance to various hemoglobinopathies, especially thalassemia and sickle cell disease. For our experimental system, we are using the K562 human leukemic cell line, as well as peripheral blood from individuals with sickle cell disease. We are studying the effects of short-term and long-term exposure of these cells to S-azacytidine and hemin on their phenotype and the factors that control globin gene transcription. Adult beta-mRNA expression remains undetectable, yet we have found a constitutive level of another adult type hemoglobin, delta-mRNA, whose expression is inducible both with hemin and 5-azacytidine. Because of the close sequence homologies between the delta- and beta-globin genes, experiments are underway to examine whether changes in the delta-promoter sequence may alter important protein binding sites and thereby result in the low levels of delta-globin gene expression. A 500 bp region 5' to the delta globin CAP site has been cloned into a chloramphenicol acetyl transferase (CAT) reporter plasmid. Preliminary studies demonstrate that this reporter system when transfected into K562 give quantitatively similar results (when compared to beta-CAT and epsilon-CAT) as were obtained using S1 nuclease mapping. Identification of these putative protein(s) binding sites may not only provide important information on the regulation of the minor hemoglobin synthesis but may allow for the characterization of trans-acting factor(s) responsible for beta-globin gene expression. Development of this functional assay system utilizing the various CAT constructions may facilitate such identification.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25059-05 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Trans-activating Factors and Globin Gene Expression: A Direct Approach**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Harish Dave

Visiting Fellow

LCB, NIDDK

Others: Alan N. Schechter

Chief

LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Humans undergo two developmental switches in their hemoglobin phenotype. The embryonic to fetal switch early in gestation and the fetal to adult switch around the time of birth. The K562 human leukemia cell line expresses all globin genes other than the adult beta-globin. Previous work from this laboratory has shown that the K562 beta-globin gene functions normally in a heterologous expression system. Elucidation of the mechanism of failure of beta-globin gene expression in K562 cells may provide an insight into globin gene expression and switching in normal erythroid cells.

A stable transfectant assay has been established to study the localization of sequences conferring tissue specificity to the upstream region of globin genes. We have shown that the 104 base pairs 5' of the cap site of the epsilon-globin gene are sufficient for tissue specific expression.

Stable transformants of K562 cells containing integrated constitutively expressing HTLV-1 TAX<sub>1</sub> genes have been developed. These cells display a stimulation of  $\alpha$ ,  $\zeta$ ,  $\epsilon$  and  $\gamma$  globin genes. This is correlated with increased % benzidine positivity and spectrophotometrically measured hemoglobin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25060-05, LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**In Vitro Transcription of Human Globin Genes With K562 Nuclear Extracts**

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

**PI: Yuko Wada Visiting Associate LCB, NIDDK****Others: Constance T. Noguchi Research Physicist LCB, NIDDK**

## COOPERATING UNITS (if any)

## LAB/BRANCH

**Laboratory of Chemical Biology**

## SECTION

**Section on Molecular Biology and Genetics**

## INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

## TOTAL MAN-YEARS:

**1.2**

## PROFESSIONAL:

**1.2**

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The K562 erythroleukemia cell line constitutively expresses low levels of embryonic and fetal but not adult hemoglobin. Hemoglobin production can be further induced by chemical stimuli such as hemin. An *in vitro* transcription assay based on the soluble cell-free nuclear extracts obtained from K562 cells has been used to investigate the regulation of human globin genes expression at a transcriptional level. To evaluate the regulatory role of 5' upstream cis-acting sequences in  $\epsilon$ -globin gene transcription, six deletion mutations on 5' upstream to the cap site ( $\Delta 65$ ,  $\Delta 104$ ,  $\Delta 177$ ,  $\Delta 274$ ,  $\Delta 453$ ,  $\Delta 535$ ) were constructed and were examined by an *in vitro* transcription assay. To detect a signal of correctly initiated transcription from the cap site, the resultant run-off transcripts were hybridized with  $^{32}\text{P}$  labeled antisense RNA probe followed by ribonuclease  $\text{T}_1$  digestion ( $\text{T}_1$  analysis). The  $\text{T}_1$  analysis of the deletion mutations demonstrated negative and positive transcriptional regulatory cis-acting elements in proximal promoter region of  $\epsilon$ -globin gene.

In order to identify the trans-acting factors related to these positive and negative regulatory cis-acting elements of  $\epsilon$ -globin gene, we fractionated K562 nuclear extracts by means of an anion exchange chromatography with step-wise ammonium sulfate concentration elution were employed to drive transcription from each deleted promoter of six mutations. The 175 mM fraction (F175) which was previously reported as the most transcriptionally active fraction showed no differential expression among the deletion mutations, suggesting the absence of specific protein factors which may interact with the negative cis-acting elements in the F175.

The  $\beta$ -globin gene could not transcribed *in vitro* by K562 nuclear extracts; however, the combination extracts of K562 nuclear extract and high-speed cytoplasmic extract of K562 cells (K562 S100) directed the transcription from  $\beta$ -globin gene promoter. In order to confirm that the signal is an accurately initiated transcription, antisense RNA probe was made and the  $\text{T}_1$  analysis was carried out. The supplement of K562 nuclear extract with K562 S100 did induce the correct initiated transcription from the cap site of the  $\beta$ -globin gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25061-05 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Isolation of Embryonic Globin Transcriptional Factors by Subtractive cDNA Cloning**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:

Yongji Wu

Visiting Associate

LCB, NIDDK

Others:

Constance T. Noguchi

Research Physicist

LCB, NIDDK

## COOPERATING UNITS (If any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.8

## PROFESSIONAL:

0.8

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transcriptional regulation of globin genes depends upon a variety of cis-acting regulatory DNA sequences and trans-acting protein or other factors. Several of the cis-acting sequences have been identified through naturally occurring and laboratory synthesized mutations or deletions. Trans-acting factors which bind to some of these cis-acting sequences have been cloned using DNA binding assays. As an alternate approach to the isolation of transcription regulatory proteins, we used a functional assay to screen a cDNA library from erythroid-like cells.

A cDNA library was constructed from K562 erythroleukemic cells.  $1.5 \times 10^5$  cDNA clones were screened for specificity to hemin induced K562 cells producing high levels of globin mRNA and hemoglobin protein. Of the 80 clones identified to be specific for induced K562 cells, 45 were found to be full length. These cDNA clones were inserted into an eukaryotic expression vector and driven by an SV40 viral promoter. To test for functional activity these clones were co-transfected with a globin promoter/reporter gene construct into HeLa cells. Six clones were found to trans-activate globin promoter activity. Four were homologous to the ferritin heavy chain, one to the ferritin light-chain, and one to no known DNA or corresponding protein sequence. The 5' region of the ferritin cDNA clones extended up to 145 bases upstream from the coding region, but did not contain the iron response element usually associated with ferritin mRNA.

These clones could activate transcription of other promoter/reporter gene constructs, but to a lesser extent than the effect on globin promoters. Transfection of the ferritin clones into K562 cells increased the endogenous epsilon globin level. We are now working on functional studies to determine if the transcriptional activation of these clones is dependent upon specific cis-acting globin DNA sequences.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25063-04 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Effect of Hydroxyurea on Fetal Hemoglobin Synthesis in Sickle Cell Patients**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	<b>Griffin P. Rodgers</b>	<b>Senior Investigator</b>	<b>LCB, NIDDK</b>
<b>Others:</b>	<b>Shu-zhen Huang</b>	<b>Visiting Associate</b>	<b>LCB, NIDDK</b>
	<b>Constance T. Noguchi</b>	<b>Research Physicist</b>	<b>LCB, NIDDK</b>
	<b>Alan N. Schechter</b>	<b>Chief</b>	<b>LCB, NIDDK</b>

COOPERATING UNITS (if any) CHB, NHLBI (A.W. Nienhuis); CB, NEI (Dr. M. Roy); BEIB (Mr. E. Walker); Depts. of Medicine, Pediatrics & Pathology, Johns Hopkins University, Baltimore, MD (Dr. G. Dover); Laboratory of Medical Genetics, Shanghai's Children's Hospital, Shanghai, China (Drs. Y. Zeng and S. Huang)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews  
☒ (b) Human tissues  
☐ (c) Neither

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have focused on pharmacological augmentation of fetal hemoglobin levels in sickle cell patients. In particular, hydroxyurea (HU), a cell-cycle specific agent that blocks DNA synthesis by inhibiting ribonucleotide reductase, has been shown to increase fetal hemoglobin (HbF) levels in some patients with sickle cell disease, although the mechanism of action remains to be defined. In order to develop protocols for effective treatment of sickle cell patients, we have studied the effects of HU administration on ten hospitalized patients treated on an escalating dose schedule for periods of three months. Of the ten patients, 7 were considered responders by virtue of at least a two-fold increase in the %F-reticulocytes and a concomitant two-fold rise in %HbF. Among the responders, HbF levels increased 2 to 10-fold, generally after a lag period of about 40 days (range - 10 to 65 days). Three patients achieved levels of HbF of 10 to 15%. The initial values of HbF, F-retics, the degree of anemia or the specific beta-globin gene haplotype were not predictive of response. Statistical analysis of the three cellular variables that determine HbF levels in these patients disclosed that HbF production, as estimated by F-retic number, accounted for about 70% of the increase in HbF. Four of the responders were retreated with optimal HU dose after a "washout" period and were found to have greater HbF responses, again occurring after a measurable lag period. Treatment with daily doses of hydroxyurea, as opposed to treatment on 4 out of seven days, or blood transfusions appear to blunt the maximal HbF response. This prolonged lag period prior to response, as well as the effects of transfusion and daily therapy suggests that mechanisms other than acute cyto-reduction with regeneration, such as changes in mechanisms controlling gamma-globin gene transcription may be operative in the increased HbF synthesis.

Should a significant sustained F-cell response in select patients while on HU, it may be possible to increase further the magnitude of the response by simultaneously administering short courses of cloned human erythropoietin or cloned granulocyte-macrophage colony stimulating factor. In this fashion, one may approach fetal hemoglobin levels consonant with those observed in the benign HbS-HPFH phenotypes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25064-04 LCB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Cytogenetic Investigations of Patients with Genetically Determined Disorders**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: **Beverly J. White** Director, Cytogenetic Unit LCB, NIDDK  
Others: **Christopher Reed** Chemist OD, CC  
**Brian Brinson** Medical Technician OD, CC

COOPERATING UNITS (if any) **Medical Genetics Program, CC (J. Mulvihill, W. Gahl, D. Parry); LN, NIA (M. Schapiro, S. Rapoport).**

LAB/BRANCH

**Laboratory of Chemical Biology**

SECTION

**Molecular Forces and Assembly (Cytogenetics Unit)**

INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

TOTAL MAN-YEARS:

**3.0**

PROFESSIONAL:

**2.7**

OTHER:

**0.3**

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In cooperation with the medical genetics program, our cytogenetic studies of 225 new clinical center patients were retrieved from a database for correlation with clinical status. Chromosomal abnormalities were most frequent in patients with admitting diagnoses of hypogonadism or infertility (22.7%) and common in patients with short stature (5.6%) and premature ovarian failure (5.6%). Of over 60 Turner syndrome patients screened, nearly 70% were 45,X and the remainder were either mosaics or had structurally abnormal X chromosomes. High-resolution karyotypes were normal in patients with primary diagnoses of metabolic disease and seizure disorder, as well as in patients with isolated aniridia, Von-Hippel Lindau disease, and Lowe's syndrome. Three of 6 patients with the Prader-Willi syndrome had deletions involving bands q11.1 to q13 of chromosome 15, consistent with other reported studies of this syndrome and with a recent report of maternal uniparental disomy in Prader-Willi patients without deletions. Complex rearrangements were found in one autistic patient and one patient with the Russell-Silver phenotype, providing clues for localization of genetic changes in these syndromes.

Several patients studied by our laboratory were described in case reports, including mosaic translocation trisomy 21 in a non-mentally retarded woman with Alzheimer's disease, an individual with 47,XXX Klinefelter syndrome and a conduct disorder, and a patient with familial carotid body tumors and extra-adrenal pheochromocytomas. Levels of expression of the fragile X chromosome in peripheral blood were determined for a group of young adult males with typical fragile X syndrome in collaboration with the NIA as part of their studies of the CNS in this disorder, which will soon be reported.

During the coming year, we plan to take part in collaborative investigations of familial premature ovarian failure and of the 18p- syndrome. These studies will emphasize localization of genes responsible for the different clinical manifestations of these syndromes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25066-04 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

AIDS: Transcriptional Regulation by TAT-Protein and LTR of HIV In Vitro

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Jiangang Yuan	Visiting Associate	LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

## COOPERATING UNITS (if any)

Kabigen, Stockholm, Sweden (Professor M. Hartmanis)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The human immunodeficiency virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS). The viral replication and high level gene expression of HIV virus require activation in trans by regulatory protein, TAT. The region of HIV needed for TAT mediated-regulation was termed the TAR (trans-acting responsive element), which is positioned between nucleotide -17 and +80 relative to the transcription initiation site and the sequence from +14 to +44 is essential for the response to TAT protein. We developed a in vitro transcriptional assay and our previously results showed that the nuclear extract of HeLa/t2 cell, which contains TAT protein, and purified recombinant TAT protein from E. Coli specifically enhanced the transcription from HIV-LTR. In the past year, we also found that the DNA sequence of TAR region was needed in vitro transcription system by using competition assay. In order to be able to provide more directly evidence for the relations between the transactivating activity of TAT protein and the TAR sequence, we have set up a gel retardation assay. The result showed that when the HeLa/TAT nuclear extract (HeLa/TAT cells was transformed with TAT cDNA) was incubated with double strand DNA probe of TAR region, the gel retardation pattern appeared a clearly extra band compared with that of normal HeLa nuclear extract. The same result was obtained from the probes which either was between nucleotide +14 to +45 or between nucleotide +19 to +45, but this extra band was disappeared when the DNA probe was between nucleotide +25 to +45. This result indicated that the DNA sequence from +19 to +25 of TAR region is essential for this extra band. we also observed that the extra band can be competed out by specific DNA competitor (cold DNA TAR sequence) and anti-TAT antibody. These results imply that the formation of this specific DNA-protein complex was due to some factor(s) which was only contained in the cells transformed by TAT gene and it was relative to the TAT protein. These results give us some information about the transcriptional regulation by TAT protein and TAR sequence. We are now going to develop other assays to screen potential inhibitors of TAT activity, prevention or treatment of AIDS.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25068-04 LCB

## PERIOD COVERED

**October 1, 1989 to September 30, 1990**

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Regulation of Globin Gene Expression by Upstream Positive Control DNA Sequences**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

**PI: Moshe Mittelman****Visiting Associate****LCB, NIDDK****Others: Patricia Berg  
Alan N. Schechter****Senior Staff Fellow  
Chief****LCB, NIDDK  
LCB, NIDDK**

## COOPERATING UNITS (# any)

## LAB/BRANCH

**Laboratory of Chemical Biology**

## SECTION

**Section on Molecular Biology and Genetics**

## INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

## TOTAL MAN-YEARS:

## PROFESSIONAL

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**This project has been terminated.**

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER <b>Z01 DK 25069-02 LCB</b>
PERIOD COVERED <b>October 1, 1989 to September 30, 1990</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Coordinated Expression of Human Beta Sickle Antilles and Human Alpha Globin in Transgenic Mice</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
<b>PI:</b>	<b>Frank Shafer</b>	<b>NRSA Fellow                      LCB, NIDDK</b>
<b>Others:</b>	<b>Zi Yao Liu</b>	<b>Visiting Associate              LCB, NIDDK</b>
	<b>Constance T. Noguchi</b>	<b>Research Physicist              LCB, NIDDK</b>
	<b>Alan N. Schechter</b>	<b>Chief                                LCB, NIDDK</b>
COOPERATING UNITS (if any) <b>DMNB, NINDS (Drs. S. Karlsson and B. Dropulic); University of South Carolina, Columbia, S.C. (Dr. M. Dewey); Nippon University, Tokyo (Dr. N. Uyesaka)</b>		
LAB/BRANCH <b>Laboratory of Chemical Biology</b>		
SECTION <b>Section on Molecular Biology and Genetics</b>		
INSTITUTE AND LOCATION <b>NIDDK, NIH, Bethesda, Maryland</b>		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
<b>1.5</b>	<b>1.5</b>	<b>0</b>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Sickle cell anemia is caused by a single base-pair substitution in codon 6 of the human <math>\beta</math>-globin gene resulting in a mutant hemoglobin molecule. Hemoglobin S (HbS) polymerizes when deoxygenated and causes erythrocytes to deform and take on a characteristic "sickled" shape. An animal model for sickle cell disease will not only allow for the analysis of in vivo erythrocyte sickling and for more detailed study of the pathophysiology of the disease, but will permit the development of potential therapeutic modalities to treat the disease and prevent the accompanying complications. Previous attempts to develop such a transgenic model have failed to achieve an anemic animal with morphologic erythrocyte changes, accompanying histopathological changes and tissue damage as evidence of phenotypic expression of the disease.</p> <p>Using two novel approaches, we may be able to upregulate the expression of the exogenous alpha- and beta-like globin genes in a coordinated fashion and increase the propensity toward intracellular polymerization resulting in a pathologic transgenic phenotype. The variant sickle cell gene that we are using is hemoglobin S-Antilles, a2b2(6glu-&gt;val,23val-&gt;ile). This double mutant variant polymerizes more readily than sickle hemoglobin and results in disease manifestations in human heterozygous carriers of the disease. Constructs have been made using the human <math>\beta</math> S-Antilles and a2 genes tandemly linked downstream of either two or four erythroid specific DNase I super-hypersensitive (HS) sites. These constructs have been injected into fertilized mouse embryos. Using isoelectric focusing, blood samples have been analyzed for the presence of human hemoglobin. Screening has demonstrated that three founder and two F2 mice are expressing human hemoglobin at levels of 15-20% of endogenous mouse hemoglobin. Quantitation of human and mouse globin chains has been determined by denaturing Triton-urea polyacrylamide gel electrophoresis. Transcription analysis is being performed using RNase protection assays and gene copy number has been determined by Southern analysis. Data on erythrocyte functional properties will include morphological analysis, oxygen affinity curves, hematological values and rheological studies. Further cross breeding of these animals should result in a line of transgenic animals manifesting the sickle cell phenotype.</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 25070-02 LCB

PERIOD COVERED

**October 1, 1989 to September 30, 1990**

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Analysis of the Epsilon Globin Gene Flanking Sequences**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	<b>Natalya Merezinskaya</b>	<b>Visiting Fellow</b>	<b>LCB, NIDDK</b>
<b>Others:</b>	<b>Constance T. Noguchi</b>	<b>Research Physicist</b>	<b>LCB, NIDDK</b>
	<b>Alan N. Schechter</b>	<b>Chief</b>	<b>LCB, NIDDK</b>

COOPERATING UNITS (if any)

LAB/BRANCH

**Laboratory of Chemical Biology**

SECTION

**Section on Molecular Biology and Genetics**

INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

TOTAL MAN-YEARS:

**1.0**

PROFESSIONAL:

**1.0**

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews  
☒ (b) Human tissues  
☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The globin gene family has been serving for a long time as a model for studying tissue-specific and developmental stage-specific regulation of expression. The K562 human erythroleukemia continuous cell line is a useful tool for examining globin gene expression as these cells express embryonic epsilon - and fetal gamma -, but not adult beta-globin genes. It has been shown that regulation of expression of globin gene family involves cis-acting DNA sequences in the vicinity of the transcription initiation site, and those play an important role in constitutive expression of the genes, as well as more upstream and downstream sequences which are thought to be necessary for tissue and developmental specific regulation. Although the role and the structure of different DNA sequences and DNA-binding proteins have been investigated very intensively, the molecular mechanisms governing developmental switching and tissue specific regulation of globin genes are still unknown. Our goals are to define functionally active DNA sequences in 5' flanking region of human epsilon- globin gene, to show the specific interaction of regulatory protein(s) with these regions, and to isolate and to characterize these specific DNA-binding protein(s).

Functional analysis of regulatory role of  $\epsilon$ -globin gene promoter region will be performed by deletion mutations using luciferase as a reporter gene. The significance of 5' flanking DNA sequences in transcriptional regulation will be further proven by site-directed mutagenesis. Experiments leading to the detection of protein interaction site(s) will include DNase footprinting and gel retardation assays. Finally, attempts will be undertaken to isolate and characterize regulatory protein(s) which specifically interact with DNA sequences of the 5' flanking epsilon-globin region.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25071-02 LCB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Trans-Regulation of Human Globin Gene**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	<b>Robert H. Broyles</b>	<b>Guest Worker</b>	<b>LCB, NIDDK</b>
<b>Other:</b>	<b>Antonello Punturieri</b>	<b>Visiting Fellow</b>	<b>LCB, NIDDK</b>
	<b>Patricia Berg</b>	<b>Senior Staff Fellow</b>	<b>LCB, NIDDK</b>
	<b>Alan N. Schechter</b>	<b>Chief</b>	<b>LCB, NIDDK</b>

COOPERATING UNITS (if any)

LAB/BRANCH

**Laboratory of Chemical Biology**

SECTION

**Section on Molecular Biology and Genetics**

INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

TOTAL MAN-YEARS

**0.7**

PROFESSIONAL

**0.7**

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Understanding the molecular regulation of developmental hemoglobin switching would be useful for increasing fetal hemoglobin in adult humans with sickle cell anemia and other hemoglobinopathies, a manipulation likely to alleviate the clinical manifestations of these diseases. The goal of this project is the identification and isolation of trans-acting factors that regulate hemoglobin switching, particularly protein regulatory factors that bind to the 5' noncoding regions of the human beta globin gene.

Hypothesis: The observation that K562 cells, and embryonic erythroid cells, have a large amount of ferritin but do not express adult beta globin, whereas adult erythroid cells have opposite characteristics, led me to hypothesize a link between the regulation of iron metabolism and regulation of globin genes, i.e., that a protein involved in the production of ferritin is a repressor of adult beta globin expression in K562 cells.

Results: A protein partially purified from K562 nuclear extracts binds the adult beta globin Rsa fragment (-223 to -129) which contains a putative positive control region (5' portion) and (3') possible negative regulatory sequences. Gel shift assays show that the protein binds the 3' portion of the Rsa fragment (-165/-129), a region that contains a DNA version of the consensus hexanucleotide (CAGTGN) of an iron responsive element (IRE), rather than the 5' positive control region (-233/-188). This protein, which is provisionally named locus repressor protein, or LRP, is a ferritin-like polypeptide or ferritin subunit as shown by its with anti-ferritin antisera and its Ferritin-like physical characteristics (heat- and proteinase K-resistance), and is prominent in K562 and -ela, but not adult chicken erythroid or MEL, cell nuclear extracts. Protected experiments will further characterize this LRP and elucidate its binding sites. In vivo footprinting will reveal DNA sites that are proteinoccupied in embryonic and adult erythroid cells.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25072-02 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Transcription of Erythropoietin

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W. David Hankins	Guest Worker	LCB, NIDDK
Others:	F. Xu	Guest Worker	LCB, NIDDK
	Kyung Bae	Guest Worker	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

## COOPERATING UNITS (if any)

Kidney Section, NIDDK (Drs. Gary and Lillian Striker); AFRRI, Bethesda, Maryland  
(Dr. T. MacVittie)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been combined with Z01 DK 25073-02 LCB

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25073-02 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**The erythropoietin receptor and genetic control red cell development**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: **W. David Hankins**

Guest Worker

LCB, NIDDK

Others:

**Feng-sheng Xu**

Guest Worker

LCB, NIDDK

**Donna Williams**

Guest Worker

LCB, NIDDK

**Yuko Wada**

Visiting Associate

LCB, NIDDK

**Kyung Chin**

Biologist

LCB, NIDDK

**Constance T. Noguchi**

Research Physicist

LCB, NIDDK

**Alan N. Schechter**

Chief

LCB, NIDDK

## COOPERATING UNITS (if any)

**Department of Biology, Massachusetts Institute of Technology, Cambridge, MA****(Dr. A. D'Andrea)**

## LAB/BRANCH

**Laboratory of Chemical Biology**

## SECTION

**Section on Molecular Biology and Genetics**

## INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

## TOTAL MAN-YEARS

**3.0**

## PROFESSIONAL

**3.0**

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Erythropoietin, a glycoprotein synthesized by the kidney, is the primary regulator of erythropoiesis and can stimulate erythroblast proliferation and differentiation. The cloning of erythropoietin has led to the way for production of the recombinant hormone and has been useful for the treatment of anemic patients with kidney failure. Less is known about the erythropoietin receptor which has been recently cloned from the mRNA of murine erythroleukemia cells. We have isolated a genomic clone containing the human erythropoietin receptor from a human placental library using a 400 bp probe from the 5' coding region of the murine erythropoietin receptor cDNA. The erythropoietin receptor gene is contained within 14 kb of genomic DNA inserted into a lambda phage vector. The coding region is localized within about 6 kb and is interrupted by several intervening sequences ranging in size from 79 bp to 1 kb. The intervening sequences are rich in Alu-related sequences. Partial sequence analysis of the coding region has enabled us to synthesize several oligonucleotide primers which were useful in generating PCR fragments from cDNA prepared from OMC1 cells, a human erythroleukemia cell line with high levels of the erythropoietin receptor on its surface. With the cloning of the genomic human erythropoietin receptor, it will now possible to study the transcriptional control of the erythropoietin receptor, its specific binding to erythropoietin and the process of signal transduction upon binding of erythropoietin at the cell surface.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25074-02 LCB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Mechanism(s) of Enhanced Gamma Globin Gene Expression in Patients**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	<b>Griffin P. Rodgers</b>	<b>Senior Investigator</b>	<b>LCB, NIDDK</b>
<b>Others:</b>	<b>Myung Nam</b>	<b>NRSA Fellow</b>	<b>LCB, NIDDK</b>
	<b>Pat Berg</b>	<b>Senior Staff Fellow</b>	<b>LCB, NIDDK</b>
	<b>Alan N. Schechter</b>	<b>Chief</b>	<b>LCB, NIDDK</b>

COOPERATING UNITS (if any)

LAB/BRANCH

**Laboratory of Chemical Biology**

SECTION

**Section on Molecular Biology and Genetics**

INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

TOTAL MAN-YEARS:

**0.8**

PROFESSIONAL:

**0.8**

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several lines of clinical and experimental evidence suggest that elevated levels of fetal hemoglobin (HbF) may improve the clinical course of individuals with sickle cell disease and beta thalassemia. A number of cytotoxic drugs have been shown to enhance gamma globin synthesis (and HbF levels) in experimental animals and patients with hemoglobinopathies, although the mechanism of action of these agents have yet to be clearly delineated. The K562 human erythroleukemia cell line has been shown to express constitutively low levels of embryonic and fetal but not adult hemoglobin, and can be reversibly induced to preferentially increase gamma globin gene expression in response to hydroxyurea. We are therefore using the K562 cell as a model system to understand the mechanism by which hydroxyurea (and similar agents) induces fetal hemoglobin synthesis. K562 cells have been grown in the presence of 25mM hemin and 0, 25, 50, 100 and, 150 mM hydroxyurea. Nuclear extracts have been prepared from these and other non-erythroid (control) cells. A 420 bp fragment 5' of the normal  $\gamma$  gene has been subcloned into an expression vector, and has been used to examine the differential binding characteristics of the nuclear protein extracts. We will employ gel-retardation electrophoresis, DNA-footprinting (DNase I and methylation protection), and ion exchange and affinity chromatography to investigate the interaction of both specific and non-specific protein/ $\gamma$  gene promoter DNA. Our preliminary results suggests that extracts obtained from hydroxyurea-induced K562 cells contains an activity not found in uninduced or hemin-induced (K562) extracts that specifically binds to the region extending from -139 to -260 of the  $\gamma$  CAP site. Furthermore, using synthetic oligonucleotides, we have sublocalized the binding site to the region between -190 and -160 of the  $\gamma$  globin gene promoter. It is hoped that the further identification, characterization and purification of this putative binding protein(s) would not only extend the current knowledge of the molecular basis of the fetal to adult "switch", but also suggest a novel pharmacological approach to the reversal of this switch in several clinically significant hemoglobinopathies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25075-02 LCB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production and Characterization of HIV TAT

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Tiee-Cherng Shieh	Visiting Fellow	LCB, NIDDK
Others:	Jian-gang Yuan	Visiting Fellow	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief,	LCB, NIDDK

COOPERATING UNITS (if any)

Kabigen AB, Stockholm, Sweden (Dr. M. Hartmanis), LCP, NIDDK,  
(Dr. A. Gronenborn), University of Padova, Italy (Dr. C. DiBello)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The human immunodeficiency virus type I (HIV-1) genome encodes the regulatory protein Tat (trans-acting transcription factor) which is a powerful trans-activator of gene expression at one or more levels and is required for efficient virus growth. Tat is 86 amino acid long, contains a cysteine rich region (from residue 22 to 37) which enables Tat to form a metal linked dimer, and it also includes a highly basic region (from residue 49-60) which is characteristic of some nuclear DNA binding proteins. During the past year, we have been able to express Tat protein in E. Coli cells by using a unique E.Coli expression system in which the Tat protein coding region was fused to the coding region for the IgG binding domain of protein A (designated Z). The coding region was altered so that chemical cleavage could be achieved between the Tat protein and Z-Z-poly peptide. By using IgG-Sepharose, gel-filtration (HPLC) chromatography, we have been successful in obtaining 15 mg of purified Tat protein. Sodium dodecyl sulfate gel electrophoresis, reverse phase HPLC, amino acid composition analysis, amino-terminal polypeptide sequencing was used for biochemical characterization. Biological or functional activity was assayed using an in vitro transcription system. Polyclonal antibodies were generated against three synthetic peptides (including regions 1-22, 53-72, 73-86 of the HIV-Tat protein sequence) and full-length recombinant Tat protein. All antibodies were successful in blocking Tat trans-activation of the HIV-LTR promoter in the in vitro transcription assay. Furthermore anti-peptide(53-72) cross reacted more strongly with the Tat protein than the other two peptides suggesting that the 53-72 region may be on the surface of the Tat molecule. We are attempting to refold the Tat protein with thiol reagents and varying renaturing conditions to facilitate the study of the biologically relevant three-dimensional conformation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25076- 01 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Trans-acting Factor(s) Controlling Epsilon Globin Gene Expression**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Betty Peters IRTA Fellow LCB, NIDDK

Others: Yuko Wada Visiting Associate LCB, NIDDK  
Constance T. Noguchi Research Physicist LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

From the third week of gestation to the second month of gestation the yolk sac is the major site of erythropoiesis. Embryonic globin chains (epsilon and zeta) are synthesized in the yolk sac. At ten weeks of gestation the site of erythropoiesis switches to the liver and the major globin chains synthesized are alpha and gamma globin. During the perinatal period the site of erythropoiesis switches to be bone marrow and alpha and beta globin are the predominant globin chains synthesized. The developmental switches that occur in globin chain synthesis are used as a model to study spatial and temporal regulation of gene expression.

Minimal globin gene promoters contain highly conserved sequences (TATA, CCAAT, CCAAC) which are required for efficient globin gene transcription. These sequences are also present and required for efficient transcription of many non-globin genes. These sequences and trans-acting factors binding to them play a role in regulating globin gene expression. Because they are highly conserved in all globin gene, it is likely that additional elements are required for spatial and temporal regulation of globin gene expression.

Functional studies in which deletion mutants of the epsilon globin gene (an embryonic globin gene) promoter have been transfected and transiently expressed in K562 cells (a cell line that produces embryonic and fetal globin chains) and HeLa cells (a cell line that does not produce hemoglobin) have identified a region of the epsilon globin gene promoter (epsilon -274 to -392) which is a silencer. This region is upstream of the minimal promoter.

Anion exchange chromatography of K562 cell nuclear extract has been used to separate a fraction that inhibits transcription of the epsilon globin gene. A mobility shift assay has identified a factor in this fraction that binds to a region of the epsilon silencer and may be important in regulating temporal and spatial expression of the epsilon globin gene. Additional anion exchange chromatography, gel filtration and affinity chromatography will be conducted in order to purify this and other factors regulating globin gene expression. Once purified the functional activity of these factors will be tested.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25077-01 LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) **B-Globin Gene Expression in Patients with Different Types of B-Thalassemia Mutation**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Shu-zhen Huang, M.D.

Visiting Associate

LCB, NIDDK

Others: Griffin P. Rodgers  
Alan N. SchechterSenior Investigator  
ChiefLCB, NIDDK  
LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The  $\beta$ -thalassemias represent a heterogeneous group of diseases, caused by different point mutations on the  $\beta$ -globin locus. However, very little is known about the mRNA transcriptional level for each of these mutant alleles. To gain insight into this area, we have developed a simple method using the enzyme reverse transcriptase and the polymerase chain reaction to amplify cDNA copies of reticulocyte mRNA so as to quantitate the relative and absolute amounts of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globin mRNA in nucleated erythroid cells from these individuals. Primers were chosen to optimize resolution and quantitation of the DNA bands separated on agarose gel electrophoresis. Quantitation was performed by scintillation counting or density scans of DNA bands stained with ethidium bromide. In our method normal individuals had a ratio of  $\alpha/\beta$  mRNA of  $1.14 (\pm 0.05)$  by scintillation counting and  $1.33 (\pm 0.04)$  by the densitometric analysis. Five patients with  $\beta^0$ -thalassemia had  $\alpha/\beta$  ratios greater than 14.0 by scintillation counting and greater than 100.0 by densitometry. Two patients with  $\beta^+$  thalassemia major also had elevated ratios of  $\alpha/\beta$  mRNA. Conversely, decreased  $\alpha/\beta$  mRNA ratios were found in patients with  $\alpha$ -thalassemia. Patients with  $\beta^0$ -thalassemia also had a demonstrable increase in their  $\gamma$  mRNA as measured in comparison to  $\alpha$  mRNA. Current studies in progress are aimed at using this PCR techniques to quantitate in absolute terms the amounts of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globin mRNA in nucleated erythroid cells. Having accomplished this goal, our efforts will be turned to examining the generality of our approach through the study of  $\beta$ -thalassemia patients with known mutations, as well as those in whom the mechanisms of the thalassemic phenotype has yet to be determined. Thus, the overall objective is to elucidate the relationship(s) between different mutations and the corresponding level of  $\beta$ -globin gene expression. On this basis, we will contribute to the specific knowledge of the genetic heterogeneity of  $\beta$ -thalassemia as well as provide new information on gene structure and function in eukaryotic cells.



ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research in the Laboratory of Chemical Physics is primarily concerned with the investigation of problems in molecular and cellular biophysics. A variety of spectroscopic techniques are employed in these investigations, including nuclear and electron magnetic resonance, Raman and infrared spectroscopies, electric-field-induced linear dichroism, ultraviolet and visible microspectrophotometry, and time-resolved optical spectroscopy using nanosecond lasers. There is also a major effort in theoretical studies to complement the experimental work, including both analytic methods and the use of high speed computers in large scale calculations. The systems under study include nucleic acids, proteins, intact and model membranes, retinal photoreceptors, and various small prototypical biological molecules. Current research focusses on: the development of new methods in NMR; the structure of proteins in solution by multi-dimensional NMR; the structure and dynamical behavior of nucleic acids; conformational, dynamical, and functional characteristics of model membrane systems; the dynamics of ligand binding and conformational changes in proteins; theoretical analysis of kinetics and dynamics in macromolecules; computer simulations of atomic motions in proteins; the molecular mechanism of excitation in photoreceptor cells and ionic processes in cell membranes; the gelation of hemoglobin S and its relation to the pathophysiology of sickle cell disease; the analysis of excited electronic states of polyenes in the vapor phase and in molecular beams; and the asymmetric synthesis and structure of small bioorganic molecules. The following gives a brief summary of the principal findings over the past year.

Bax and colleagues have made several major advances in the application of NMR to the determination of the structure of proteins in solution. New approaches have been developed for making resonance assignments in proteins with molecular weights as high as 25 kDa. To reduce resonance overlap the spectra are spread in three independent frequency dimensions. With this methodology complete backbone resonance assignments have been obtained for the protein calmodulin (16.7 kDa) in a very straightforward manner. Using an analogous approach, complete proton and carbon resonance assignments were made for the amino acid side chains of both calmodulin and interleukin-1 $\beta$ . A newly developed heteronuclear 3D NOESY experiment permits determination of a very large number of interproton distances, which form the basis of structure calculations. It has also been demonstrated (in collaboration with Clore and Gronenborn) that recording of four-dimensional NMR spectra is feasible, and can be very useful for assigning specific NOE interactions.

Ikura, Kay, and Bax have shown that the recording of 3D  $^{13}\text{C}$ -edited NOESY spectra for proteins uniformly enriched with  $^{13}\text{C}$  can be performed with high resolution in a reasonable amount of time. Kay, Ikura, and Bax have developed a more efficient strategy for obtaining J connectivity information for amino acid side chains in larger proteins that uses a three step magnetization transfer. Ikura, Kay and Bax have developed four new heteronuclear triple resonance 3D NMR experiments that can provide complete backbone resonance assignments of larger proteins in an automated manner. Ikura, Krinks, Torchia, and Bax have developed an efficient approach for obtaining a substantial number of backbone resonance assignments from a single sample that contains a number of residues with varying amounts of  $^{13}\text{C}$  and  $^{15}\text{N}$ .

enrichment, using conventional 2D NMR. Zhu and Bax have demonstrated an improved way for applying linear prediction to multi-dimensional NMR that results in significantly increased resolution compared with the conventional use of linear prediction.

Clore Gronenborn, and colleagues have used NMR to solve the complete three-dimensional structure of several proteins in solution. These include the cytokine interleukin-8, interleukin-1 $\beta$ , human thioredoxin and the zinc finger of a human enhancer binding protein. The structure of interleukin-8 is based on a total of 1880 experimental distance restraints and 362 torsion angles. The molecule is a dimer and has a motif in which two symmetry-related  $\alpha$ -helices, approximately 2.4 nm long and separated by about 1.4 nm, lie on top of a six-stranded antiparallel  $\beta$ -sheet platform derived from two three-stranded Greek keys, one from each monomer unit. It is suggested that the two  $\alpha$ -helices form the binding site for the cellular receptor, and that the specificity of interleukin-8, as well as that of a number of related proteins involved in cell specific chemotaxis, mediation of cell growth and inflammatory response, is achieved by the distinct distribution of charged and polar residues at the surface of the helices. The NMR structure was used as a starting model for the solution of the X-ray structure of the crystal by Wlodawer and coworkers (NCI). Although the structures are very similar there are several interesting differences, including the conformation of one of the loops and the relative orientation of the two subunits resulting from an unusual geometry for the  $\beta$ -sheet in the crystal structure. These findings indicate that interleukin-8 has the potential to undergo a conformational change which could be of functional significance in the binding to the receptor.

The structure of interleukin-1 $\beta$  has been solved by Clore and Gronenborn at low resolution, and consists of 12  $\beta$  strands arranged in three pseudo-symmetric topological units (each consisting of 5 anti-parallel  $\beta$ -strands). 11 bound water molecules have been located which are involved in hydrogen-bonding interactions that stabilize the conformation and thus constitute an integral part of the structure. The backbone dynamics of interleukin 1 $\beta$  have also been investigated. All the residues exhibit very fast motions (<20-50 ps), 32 (of the 153) residues display motions on a time scale of 0.5-4 ns, slightly less than the overall rotational correlation time of the protein of 8.3 ns, and another 42 residues are characterized by some sort of motion on the 30 ns to 10 ms time scale.

Clore, Gronenborn, and Forman-Kay have solved the structure of the recombinant human thioredoxin. The structure consists of a five-stranded  $\beta$ -sheet surrounded by one short and four long  $\alpha$ -helices, with an active site protrusion containing the two redox active cysteines. The overall structure is similar to the crystal structure of oxidized and the NMR structure of reduced thioredoxin from *E. coli*. This high degree of homology contrasts markedly the rather moderate degree of amino acid sequence homology of only 25%.

Clore, Gronenborn, and Omichinski have solved the structure of a 30 residue synthetic peptide containing the carboxyterminal 'zinc finger' motif of a human enhancer binding protein. The structure consists of two irregular ant-parallel  $\beta$ -strands connected by an atypical turn (residues 3-12) and a classical  $\alpha$ -helix (residues 14-24). The zinc is tetrahedrally coordinated to the sulfur atoms of two cysteines and to the N $_{\epsilon 2}$  atoms of two histidines. The presence of structural homology with a proteinase inhibitor indicates that the 'zinc finger' motif is not unique to a class of DNA binding proteins, but may represent a general folding motif found in a variety

of proteins irrespective of their function.

Gronenborn and Clore, in collaboration with Wilson (NCI) and Wingfield and Stahl (OD) have expressed the RNase H domain of HIV reverse transcriptase. The protein has been produced in large quantities in *E. coli* and purified for structure determination by NMR using a wide variety of 3D heteronuclear double and triple resonance experiments.

Becker and Khetrpal have been developing NMR methods to study the structure of small molecules oriented in liquid crystals. The approach is to use solid-state NMR spinning techniques with deuteration of the liquid crystal solvent to substantially reduce the background contributions.

Charney and Chen are investigating the structure and dynamical properties of DNA in solution using the technique of electric-field-induced dichroism. The electro-optic properties of random sequence fragments, as well as fragments containing the potentially biologically-important CAC/GTG triplet, have been prepared and their electro-optical properties are being studied in order to determine the effect of base sequence on the flexibility of DNA.

Levin, Lewis and coworkers are using Raman and infrared spectroscopy to investigate the structural and dynamical properties of both model and intact biological membranes. Fourier-transform Raman spectroscopic techniques have been developed which afford several advantages over conventional Raman methods. This technique has been used to study unsaturated lipid systems, which have led to the development of a model in which the conformational changes of integral membrane proteins can be modulated by a variation in a lipid matrix fractional volume parameter. The Fourier-transform Raman technique was also used in elucidating thermotropic and acyl chain packing characteristics of model phosphatidylcholine bilayer assemblies whose variation in sn-2 chain unsaturation mimics naturally occurring lipids in biological membranes. These studies showed that microdomains are formed in which the lipid molecules pack in a manner that maximizes primarily the van der Waal's interactions between saturated hydrocarbon sn-1 chains. A regulation of acyl chain unsaturation, and hence the degree of microdomain heterogeneity, provides a mechanism by which organisms can control bilayer properties responsible for optimizing the various membrane functions associated with integral proteins.

Hofrichter, Henry, and Eaton are using time-resolved absorption spectroscopy with nanosecond lasers to study the quaternary transition kinetics of hemoglobin in order to understand the mechanism of this important conformational change. The enthalpies and entropies of activation for trout hemoglobin calculated from transition state theory for the T to R quaternary conformational change of the zero-liganded molecule were found to be much more similar to the equilibrium enthalpy and entropy changes than the corresponding values for the R to T transition. From this result it was concluded that the transition state is much more R-like than T-like. This concept explains the finding of a linear free energy relation between the rates and equilibrium constants for human hemoglobin. Time-resolved transient absorption spectroscopy is also being used to measure the kinetics of ligand rebinding and conformational changes subsequent to photodissociation of the carbon monoxide complex of a hemoglobin hybrid where cobalt has been substituted for iron in the  $\alpha$  chain porphyrins. Progress curves for the singly and

doubly-photodissociated molecules show identical geminate rebinding phases, indicating that there is no interaction between  $\beta$  subunits in times less than about 300 ns. In contrast to myoglobin, where the kinetics of ligand rebinding to the single binding site are described by two relaxations, a minimum of 5 relaxations are required to fit the data for the singly-photodissociated molecule, implying that there are at least 2-3 conformational relaxations that influence the ligand rebinding rates. Models in which the subunits within each quaternary structure have two tertiary conformations with different geminate rebinding rates explain the results better than models in which there is only a single geminate rebinding rate for each quaternary structure.

Eaton and Henry, in collaboration with Mozzarelli (University of Parma), are measuring the oxygen binding curves of single crystals of hemoglobin in the T quaternary structure using a microspectrophotometer. These studies show that the crystals bind oxygen non-cooperatively with no Bohr effect, a result that has several important implications for the molecular mechanism of cooperativity in hemoglobin.

Christoph, Hofrichter, and Eaton are studying the origin of the wide variety of shapes observed upon deoxygenation of sickle cells. Measurements of gelation delay times and domain density indicate that the rate of domain formation is comparable to the rate of homogeneous nucleation predicted by the double nucleation model for polymer formation. Optical micrographs of cells deoxygenated at various rates indicate that the morphology of cells is controlled by the density of polymer domains, and, therefore, the rate of homogeneous nucleation. Thus the delay time of gelation not only determines whether intracellular polymerization will occur in vivo, but it also determines the shape of the resulting sickled cell.

Szabo and Zhou have carried out several theoretical investigations on dynamical processes. A dynamical mean field theory has been developed for analyzing fluorescence quenching experiments performed in the frequency domain. A unified theory has been formulated for reversible diffusion-influenced geminate and pseudo first order reactions. An important feature of the theory is that arbitrary initial conditions can be treated and that in each case the equilibrium limit is correctly predicted. In a related study Zhou has investigated the procedure now commonly used to simulate the bimolecular diffusion-controlled reaction rate between macromolecules and ligands, and was able to find an analytical derivation of the key equation upon which this procedure is based. Szabo has generalized his earlier model free approach on the interpretation of NMR relaxation data to incorporate internal motions on different time scales, and in collaboration with Clore and Gronenborn has shown how the existing data on proteins can be used to quantitate the time scale and amplitude of side-chain motions.

Zwanzig is theoretically investigating rate processes in disordered systems. One study dealt with diffusion in a disordered medium, and in particular with the effects of time- and space-dependent fluctuations in the diffusion coefficient. In a second investigation, a more accurate and transparent treatment has been made of Berg and Purcell's theory of diffusion controlled ligand binding to receptors randomly distributed on the surface of a cell. In a third area, a major review has been written about theoretical methods that have been invented to treat kinetic processes where rate constants can fluctuate in time (dynamical disorder).

Hagins and Yoshikami are investigating the mechanism of visual transduction. Using

intensified video microscopy and quantitative image analysis, responses of intact rod cells and of isolated outer segments to changes in external calcium have been followed with calcium-sensitive dyes in order to study calcium regulation in these cells. Agents that increase the permeability of rod plasma membranes to calcium greatly increase the sensitivity of the cytoplasmic calcium pool to changes in external calcium concentration. A more sensitive microcalorimeter has been designed for studying the fast hydrolysis of several kinds of phosphate esters that have been implicated as part of the transduction mechanism. A family of compounds based on substituted imidazoles bearing fluorine, bromine, or iodine atoms is being tested as markers for membrane potential and water content in intracellular compartments of living cells by x-ray microanalysis of freeze-dried blocks or sections. The water content of the various components of living retinas has been estimated and shown to agree with other less selective methods.

Kon is using electron paramagnetic resonance (EPR) for studying the binding of metalloporphyrins to DNA fibers oriented in the EPR magnetic field. The orientation of the porphyrin plane with respect to the helix axis can be determined from the orientation of the g tensors of the paramagnetic metal. Whereas 4-coordinate copper porphyrins intercalate between base pairs, 5-coordinate complexes bind to the groove of the double helix.

McDiarmid is carrying out optical spectroscopic studies on the electronic structure and conformation of the excited states of small polyatomic molecules that are models for more complex biological molecules. Analysis of the first allowed valence state of cyclopentadiene shows that the molecule is significantly elongated along the C=C bonds and has an excited state lifetime that increases linearly with energy. From analysis of the simultaneously measured absorption and ionization spectrum it was concluded that excitation into the highly excited states of ethylene results in photodissociation that is faster than ionization into the continuum. The best random phase quantum mechanical calculations of the one and two photon resonant intensities to the  $n=3$  Rydberg states of acetone were demonstrated to be incapable of accurately reproducing the experimental intensities.

Ziffer is synthesizing novel compounds that are potential agents for the treatment of malaria. Esters and ethers of dihydroartemisinin, a derivative of a drug currently being used, have been modified for structure-activity studies. Two products have been isolated from studies with the fungus *Beauveria sulfurescens*, which was used to introduce a hydroxyl group into the N-phenyl urethane of dihydroartemisinin. One was shown by NMR studies to contain a new hydroxyl group, present as hydroxymethyl, and still had intact the critical peroxide group required for biological activity. The structure of the second product was also determined by NMR and it was found to have been formed by a rearrangement of the peroxide group. A variety of derivatives of the first compound will be prepared and tested for their activity against cerebral malaria.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29001-18-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular dynamics and vibrational characteristics of membrane assemblies

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ira W. Levin Research Chemist LCP-NIDDK

Others:	E. Neil Lewis	Visiting Associate	LCP-NIDDK
	Burton J. Litman	Special Volunteer	LCP-NIDDK
	James L. Slater	IRTA	LCP-NIDDK
	Paul Harmon	IRTA	LCP-NIDDK
	Mark Devlin	IRTA	LCP-NIDDK

## COOPERATING UNITS (if any)

R. Adams, LCP-NIDDK; Clifford J. Steer, Medical School, Univ. of Minn.; C. Huang, School of Medicine, Univ. of VA; James S. Vincent, Univ. of MD.; Sherwin Straus, FDA

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Vibrational Raman and infrared spectroscopy are used to probe the dynamical, conformational, functional and thermodynamic properties of both model and intact membrane assemblies. Emphasis is placed on elucidating lipid-lipid and lipid-protein interactions within the bilayer aggregate. For example, both Fourier-Transform (FT) Raman and visible laser Raman techniques were employed in elucidating the thermotropic and acyl chain packing characteristics of model phosphatidylcholine bilayer assemblies whose variation in sn-2 chain unsaturation mimics naturally occurring lipids in biological membranes. The noncooperative phase transition behavior for the multilamellar lipid systems containing four and six double bonds in the unsaturated chain (namely, PAPC and PDPC) was interpreted in terms of specific acyl chain clusters. That is, microdomains are formed in which the lipid molecules pack in a manner that maximizes primarily the Van der Waal's interactions between saturated hydrocarbon sn-1 chains. A regulation of acyl chain unsaturation, and hence the degree of microdomain heterogeneity, provides a mechanism by which organisms can control the bilayer properties responsible for optimizing the various membrane functions associated with integral proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29002-17 LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry of natural compounds, and synthetic organic chemistry

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ulrich Weiss (deceased) Research Chemist LCP-NIDDK  
(Scientist Emeritus)

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

TERMINATED

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29005-16

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Asymmetric synthesis; structure, stereochemistry, and NMR

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. : Herman Ziffer Research Chemist LCP-NIDDK

Others: Xi-Ni Zhang Visiting Fellow LCP-NIDDK  
Yulin Hu Guest Worker LCP-NIDDK

## COOPERATING UNITS (If any)

Dr. M. Duncan, NIMH, LCS.

Dr. R. J. Highet, NHLBI, IR CH.

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.8

## PROFESSIONAL:

2.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (a1) Minors☐ (a2) Interviews☐ (b) Human tissues☒ (c) Neither

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The formidable problems encountered in developing a malaria vaccine, and the ability of Plasmodium falciparum to become resistant to new drugs, have stimulated interest in using combinations of drugs for treating this disease, which claims more than a million lives a year. One drug of current interest, artemisinin, was isolated by Chinese investigators from a traditional medicinal herb. Esters and ethers of dihydroartemisinin have been prepared by several groups for structure-activity studies of their antimalarial properties. These demonstrated that the peroxide bridge was required for pharmacological activity. In order to increase the functionality of the molecule, we investigated the ability of the fungus Beauveria sulfurescens to introduce a hydroxyl group into the N-phenyl urethane of dihydroartemisinin. Two products were isolated from these studies. One was shown by  $^1\text{H}$ ,  $^{13}\text{C}$  and 2D nmr studies to contain a new hydroxyl group, present as a hydroxymethyl, and still had intact the peroxide group required for biological activity. The structure of the second product was also determined by nmr and it was found to have been formed by a rearrangement of the peroxide group. A variety of derivatives of the first compound will be prepared and tested for their activity against cerebral malaria.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01-DK-29006-20-LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The structure and dynamics properties of macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Elliot Charney

Research Chemist

LCP-NIDDK

COOPERATING UNITS (if any)

H-H Chen, George Mason University, Fairfax, VA; Rodney Harrington, University of Nevada, Reno, NV; D. C. Rau, LCB-NIDDK

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS: PROFESSIONAL

1.5

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews  
☐ (b) Human tissues  
☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Macromolecular structure, dynamics and polyelectrolyte properties of large biological polymers, in particular, polynucleotides and nucleic acids are being studied by electric-field induced dichroism and birefringence methods.

The current research is a response to the fact that the knowledge of the structural effects of specific base-pair sequences on DNA translation and replication is still at a primitive stage. Only one or two biologically significant protein-DNA complexes from which such structural effects could be inferred have been crystallized and their structure determined. Using electro-optic birefringence and dichroism, it is now possible to quantitatively explore DNA structures in solution, albeit with less resolution than x-ray diffraction of crystals, but uninhibited by the problem of forming crystalline complexes. The two principal projects currently being pursued are the structural effects of the triplet sequence CAC/GTG and the flexibility of the A form of DNA.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29007-19-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and interaction of biomolecules

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Hideo Kon

Research Chemist

LCP-NIDDK

## COOPERATING UNITS (if any)

Makoto Chikira, Chuo University, Tokyo, Japan

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Spectroscopy and Structure

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In this project, we aim at applying electron paramagnetic resonance (EPR) spectroscopy to probe structure-function relationships in biological systems and attempt to develop a new mode of application. This year, in collaboration with Dr. Makoto Chikira, Special Volunteer, we studied by EPR the binding behavior of various metalloporphyrins, metal complexes of amino acids, and metal complexes of bleomycin on DNA fiber which is held oriented in EPR magnetic field. Some of these metal complexes have photosensitive character and others can activate molecular oxygen. One objective of this project is to determine how these complexes bind to DNA, i.e., the relationship between, e.g. the porphyrin structure and the base-pair sequence specificity of binding and the geometry of porphyrins on DNA. Specific example of results obtained are: 1) Cu(4TMpyP) ---tetramethylpyridin deritive--- intercalates in DNA double helix, while Cu(4TMpyP)-imidazole complex and Cu(2TMpyP) bind to the groove of DNA double helix with the  $g_{11}$  axis, respectively, parallel and perpendicular to the groove, 2) EPR signals corresponding to both intercalated and groove-bound Co(4TMpyP) were resolved, and computer simulations of line shapes are now underway, 3) High spin Fe(4TMpyP) binds to the groove of DNA with the orientation similar to that of Cu(2TMpyP). Low spin bis-imidazole adduct of Fe(4TMpyP) binds to the DNA with the porphyrin plane being parallel to  $g_x$  axis being almost perpendicular to the DNA fiber axis. In the case of oxygenated Co(II)-bleomycin complex, the oxygen-oxygen bond is restricted to a plane perpendicular to the fiber axis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29008-19-LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electric and molecular structural investigation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ruth McDiarmid Research Chemist LCP-NIDDK

Others: Donald Jordan IRTA Fellow LCP-NIDDK  
Aharon Gedanken Special Volunteer LCP-NIDDK

COOPERATING UNITS (if any)

Leo Klasinc, Rugjer Bošković Institute, Zagreb, Yugoslavia; Yuri Panchenko, Moscow State University, Moscow, USSR; Robert Wu, Univ. Southern California, Los Angeles, CA

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The absorption spectra of the first allowed valence transition of cyclopentadiene and cyclopentadiene- $d_6$  were analyzed. The long vibrational progression was shown to be a progression in predominantly the C=C stretching coordinate. The molecule was deduced to be significantly elongated along the C=C bonds in this excited state. Its excited state lifetime was shown to be 35 fsec and to increase linearly with energy.

Superexcited states of ethylene, between the first ionization potential and 1060A, were shown, by an analysis of the simultaneously obtained absorption and ionization spectrum, to couple strongly to the excitation continuum and to photodissociate more rapidly than the molecule ionizes into the continuum.

The best random phase quantum mechanical calculations of the one and two photon resonant intensities to the  $n=3$  Rydberg states of acetone were demonstrated, by experimental measurement, to be incapable of accurately reproducing the experimental intensities.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29009- 17-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on sickle cell disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: William A. Eaton Medical Officer LCP-NIDDK

Others: James Hofrichter Research Chemist LCP-NIDDK  
Garrott W. Christoph Expert LCP-NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Macromolecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(TERMINATED - THIS REPORT IS NOW COMBINED WITH  
REPORT NUMBER Z01-DK-290010)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DK-29010-18-LCP
--	---------------------------------------

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dynamics of Proteins and Studies on Sickle Cell Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	William A. Eaton	Medical Officer	LCP-NIDDK
Others:	James Hofrichter	Research Chemist	LCP-NIDDK
	Eric R. Henry	Research Physicist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen M. Jones	I.R.T.A.	LCP-NIDDK
	Garrott R. Christoph	Expert	LCP-NIDDK

COOPERATING UNITS (if any)

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, Italy

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
☐ (b) Human tissues
☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Time-resolved optical spectroscopy with nanosecond lasers and molecular dynamics calculations are being employed to investigate ligand rebinding and conformational changes in hemoglobin subsequent to photodissociation of the carbon monoxide complex. These methods have been used to determine kinetic parameters for geminate rebinding in hemoglobin in both the R and the T quaternary structures, to measure the rate of the R to T structural change, and to measure the kinetics of the tertiary conformational changes resulting from ligand dissociation in both quaternary structures. Comparison of activation and equilibrium parameters shows that the transition state for the quaternary transition is much more R-like than T-like, explaining the linear free energy relation between quaternary rates and equilibria. The R-like transition state may be explained by a reaction path which maximizes the buried surface area. In a related project it has been shown that single crystals of hemoglobin in the T quaternary structure bind oxygen non-cooperatively with no Bohr effect, a result that has several important implications for the molecular mechanism of cooperativity.

Studies are being carried out to provide a quantitative description of the gelation of hemoglobin S that can be used for understanding the pathophysiology of sickle cell disease and the development of therapeutic agents. Measurements of gelation delay times and domain density indicate that the rate of domain formation is comparable to the rate of homogeneous nucleation predicted by the double nucleation model for polymer formation. Optical micrographs of cells deoxygenated at various rates indicate that the morphology of cells is controlled by the density of polymer domains, and, therefore, the rate of homogeneous nucleation. Thus the delay time of gelation not only determines whether intracellular polymerization will occur in vivo, but it also determines the shape of the resulting sickled cell. This information is critical to the design of an automated "sickling assay" that will make it possible to examine a large number of potentially therapeutic agents, to compare intracellular gelation and clinical severity, and to follow changes in intracellular gelation in patients on various therapeutic protocols.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29011-19-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The physics and chemistry of photoreception

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. :	William A. Hagins	Medical Officer	LCP-NIDDK
Others:	S. Yoshikami	Research Biologist	LCP-NIDDK
	F. M. Hagins	Guest Worker	LCP-NIDDK
	M. C. Foster	Research Physicist	LCP-NIDDK
	P. Ross	Research Chemist	LMB-NIDDK
	K. Spring	Research Med. Officer	LKM-NHI
	R. Tate	Computer Systems Analyst	CSL-DCRT

## COOPERATING UNITS (if any)

Sudhir Sadhu, Student Volunteer, Thomas Jefferson High School, Alexandria, VA

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
 ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

Calcium-sensitive dyes, FLO-3 and Fura-2 have been introduced into retinal rods of living frog retinas by hydrolytic transfer of lipid-soluble esters. Using intensified video microscopy and quantitative image analysis, responses of intact rod cells and of isolated outer segments to changes in external calcium ion activity have been followed in order to study calcium regulation in these cells. The hydrolysis of lipid-soluble esters of the indicator dyes is very non-uniform from cell to cell, and two classes of rods are observed: one type constituting 1-2% of all rods accumulating 5-10 times as much indicator as the other 98-99% of the rod population within a given incubation period. The two populations can be demonstrated with a variety of indicators esterified with acetoxymethyl groups. Calcium indicators in the heavily labeled cells respond only weakly to changes in external calcium activity unless the plasma membranes have been made leaky with calcium-bearing ionophores.

New pyroelectric detectors with stacked PVDF films have been prepared to increase heat sensitivity of the devices and to reject acoustic pickup. A recording amplifier that compensates for sudden heating and cooling effects caused changing the bathing solutions around tissues on the calorimeter detector has been designed and is under construction. Extension of pyroelectric methods to solution microcalorimetry is under way.

4(5) bromoimidazole has been tested as a marker for the aqueous cytoplasmic spaces in cells that are later freeze-dried for electron-probe microanalysis. Using a variety of water-loaded dextran polymers as test systems, the accuracy of estimating water content by analyzing residual Br content of the dried polymers has been confirmed. Water content of the various components of living retinas has been estimated and shown to agree with other, less selective methods. Bromoimidazole should be useful as a water marker in many other types of tissues.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29016-15-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Macromolecular dynamics and assembly reactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	James Hofrichter	Research Chemist	LCP-NIDDK
Others:	William A. Eaton	Medical Officer	LCP-NIDDK
	Eric Henry	Research Chemist	LCP-NIDDK
	Richard Lozier	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen Jones	Visiting Fellow	LCP-NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Spectroscopy and Structure

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.7

## PROFESSIONAL:

1.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Time-resolved absorption spectroscopy is used to study the dynamics of protein structural changes subsequent to excitation with short laser pulses. Molecular models for the protein dynamics are used to fit and interpret the measured data.

- A. The kinetics of ligand binding and conformational changes for **human hemoglobin (HbA)** have been studied following the **photodissociation of carbon monoxide** from the hemes. These studies have focussed on the hybrid molecule in which cobalt is substituted for iron in the alpha chains. In particular, we have focussed on obtaining sufficient data to test specific **molecular models** for the rebinding dynamics.
- B. The photocycles of **bacteriorhodopsin (bR)**, a photoactive proton pump have been investigated. Our studies of the all-trans cycle show evidence for **back reactions** in the steps which occur in less than 1 ms. Our results also suggest that a linear sequence of reversible steps is a sufficient description of the slower steps in the cycle.
- C. The components for a **new spectrometer** with time resolution less than 1 picosecond are being assembled and tested. This instrument will be capable of performing time-resolved single photon counting fluorescence experiments and pulse-probe time resolved absorption experiments.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29017-11-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Spectroscopic investigation of membrane lipids and models

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ralph G. Adams

Research Physicist

LCP-NIDDK

Others: Ira W. Levin

Research Chemist

LCP-NIDDK

## COOPERATING UNITS (if any)

Sherwin Strauss (FDA)

## LABORATORY

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Using integrated vibrational intensity techniques, our continuing research into the role of dipalmitoylphosphatidyl choline (DPPC) in human lung surfactant has been supplemented by the new approach of Fourier transform (FT) Raman spectroscopy using near infrared laser excitation. FT Raman data thus far corroborates our findings obtained from conventional Raman spectroscopy. It is yet too early to make conclusions concerning the dominant lipid/protein interactions in lung surfactant. The ability to obtain Raman spectra of native material, free from an overwhelming fluorescence signal, demonstrates that FT Raman techniques can provide an important new biophysical tool.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29019-10-LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Theoretical studies on the dynamic aspects of macromolecular function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	A. Szabo	Research Chemist	LCP-NIDDK
Others:	X. Zhou	Research Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

N. Agmon, Hebrew University

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A dynamical mean field theory has been developed for the frequency dependence of the modulation and phase angle that are monitored in frequency domain fluorometric studies of diffusion-influenced fluorescence quenching. This theory is simple to implement and should prove useful in analyzing frequency domain fluorometric data. While most bimolecular chemical reactions are reversible previous theoretical work has focused on understanding the role of diffusion on irreversible reactions. We have formulated a computationally viable and unified theory of reversible diffusion influenced geminate and pseudo-first-order reactions. Our formalism can handle arbitrary initial concentrations of reactants and in each case the equilibrium limit is correctly predicted at long times. The kinetics of a unimolecular reaction is conventionally described by solving the familiar rate equations. The validity of this macroscopic description has been investigated in a framework of a microscopic model by numerically solving the Langevin equation for a particle moving on a listable potential as a function of the activation energy and the friction. Finally, the model-free approach to the interpretation of NMR relaxation in proteins has been generalized to incorporate both slow and fast internal motions. The resulting formalism was applied to experimental data to quantitate the time scales and amplitudes of motions of NH bonds in proteins.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29020-06-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear magnetic resonance: new methods and molecular structure determination

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Ad Bax	Visiting Scientist	LCP-NIDDK
Others:	Rolf Tschudin	Electronics Engineer	LCP-NIDDK
	Lewis Kay	Special Volunteer	LCP-NIDDK
	Mitsuhiko Ikura	Visiting Associate	LCP-NIDDK
	Guang Zhu	Student Volunteer	LCP-NIDDK
	Silvia Spera	Special Volunteer	LCP-NIDDK
	Tony Sheng	Biological Aide	LCP-NIDDK

## COOPERATING UNITS (if any)

Marius Clore, Angela Gronenborn, NIDDK/LCP, Dennis A. Torchia, NIDR/LBR; Claude Klee NCI/LB.

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Nuclear Magnetic Resonance

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.9

## PROFESSIONAL:

2.1

## OTHER:

2.8

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

New approaches have been developed for making resonance assignments in larger proteins, up to about 25 kDa. The new methods do not rely on the small and frequently unresolvable homonuclear proton J coupling, but instead utilize relatively large and well resolved homo- and heteronuclear one-bond couplings. To reduce resonance overlap, it is necessary to spread the new spectra in three independent frequency dimensions. Using this new methodology, complete backbone resonance assignments were obtained for the protein calmodulin (16.7 kDa) in a very straightforward manner. Using an analogous approach, complete proton and carbon resonance assignments were made for the amino acid side chains of both calmodulin and interleukin-1 $\beta$ . A newly developed heteronuclear 3D NOESY experiment permits determination of a very large number of interproton distances, which form the basis of structure calculations, currently in progress. We have demonstrated for the first time that recording of four-dimensional NMR spectra is feasible, and can actually be very useful for assigning specific NOE interactions.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29021-05-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Conformation and dynamics of biological macromolecules

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Eric R. Henry Research Physicist LCP-NIDDK

Others: William A. Eaton Medical Officer LCP-NIDDK  
 James Hofrichter Research Chemist LCP-NIDDK  
 Anjum Ansari Visiting Associate LCP-NIDDK  
 Colleen M. Jones IRTA Fellow LCP-NIDDK

## COOPERATING UNITS (if any)

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, ItalyTotal

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Macromolecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have studied the structural and ligand-binding dynamics of two model systems for human hemoglobin, component I of trout hemoglobin and a hybrid hemoglobin with cobalt substituted for iron in the alpha subunits, by measuring time-resolved optical absorption spectra following photodissociation of carbon monoxide as a function of degree of photodissociation. For trout I hemoglobin, a global description of the temporal evolution of the spectrum of the photolyzed molecule for degrees of photodissociation ranging from 10% to 100% requires seven exponential relaxations involving ligand rebinding and/or protein conformational changes. The first two relaxations involve both geminate rebinding of dissociated ligands and tertiary structural changes in photolyzed subunits of the protein. The third relaxation has been shown to reflect the quaternary conformational change of both zero- and singly-liganded molecules from the R to the T structure. We have derived the activation energy of the R-T change in the zero-liganded molecule from the temperature dependence of the rate of this relaxation, and we have determined that the transition state in this process is energetically (and therefore probably structurally) much more similar to the R state than the T state. The cobalt-substituted hybrid hemoglobin has only two binding sites for carbon monoxide, and from the spectra measured at partial photolysis we have determined separately for the zero- and singly-liganded molecules the time courses of both ligand rebinding and protein conformational changes. We have also derived oxygen binding curves for single crystals of human hemoglobin from polarized optical absorption spectra of crystals at various oxygen pressures. We have observed that hemoglobin in the crystal binds oxygen essentially non-cooperatively and with very low affinity.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29022-03-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural studies of AIDS proteins by NMR

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Ad Bax	Visiting Scientist	LCP-NIDDK
Others:	Paul C. Driscoll	Visiting Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK
	Jim Omichinski	IRTA Fellow	LCP-NIDDK
	Bruce Grassberger	IRTA Fellow	LCP-NIDDK

## COOPERATING UNITS (if any)

Protein Expression Laboratory (Paul Wingfield, Stephen Stahl); Ettore Appella (NCI); Sam Wilson (NCI); Pat Becerra (NCI)

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Nuclear Magnetic Resonance

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Work has been initiated on a number of structural problems related to proteins derived from the HIV virus. These include RNase H domain of reverse transcriptase, nef, and proteins of the immune system, in particular interleukin-1 $\beta$  and interleukin-8. The RNaseH domain has been purified in large amounts and N-15 and C-13 labeled protein has been obtained. Current work is being focused on resonance assignment using 3D heteronuclear NMR methods. Complete assignments of interleukin-1 $\beta$  have been obtained, the secondary structure elucidated and a low resolution 3D structure determined. The high resolution 3D structure of the interleukin-8 dimer in solution has been determined. In addition we have determined the high resolution 3D structure of the zinc finger domain of a human enhancer binding protein which binds to the regulatory region in the long terminal repeat of the HIV genome.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29023-03-LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (40 characters or less. Title must fit on one line between the borders.)

Determination of Three-Dimensional Structures of Macromolecules in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientists	LCP-NIDDK
Others:	Paul Driscoll	Visiting Fellow	LCP-NIDDK
	Julie Forman-Kay	Guest Researcher	LCP-NIDDK
	Bruce Grassberger	IRTA Fellow	LCP-NIDDK
	Jim Omichinski	IRTA Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

LCP/NIDDK (Ad Bax, Lewis Kay, Attila Szabo), Protein Expression Laboratory (Paul Wingfield, Stephen Stahl); NCI (Ettore Appella, Alexander Wlodawer)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work in this laboratory has been focused on the determination of three-dimensional structures of macromolecules in solution by NMR. Methods are being developed to increase the precision with which structures can be determined, the molecular weight range of proteins that can be analysed, and the efficiency of the computational methods used to determine the structures on the basis of the NMR data. In particular, we have demonstrated the applicability of heteronuclear 3D NMR to the study of proteins in the range 15-25 kDa, and recently, we have demonstrated the utility of heteronuclear 4D NMR to assign nuclear Overhauser effects in virtually an automated manner which will permit the extension of the method to even larger proteins.

High resolution solution structures of a number proteins have been determined. These include the cytokine interleukin-8, human thioredoxin and the zinc finger domain of a human enhancer binding protein. Extensive use in these studies has been made of systematic conformational searches to obtain stereospecific assignments and torsion angle restraints which have enabled us to obtain structure of much greater precision and accuracy than was heretofore possible. The typical accuracy attainable is 0.3-0.4 Å for the backbone atoms and 0.4-0.6 Å for the internal side chains.

Work is in progress on determining the solution structures of a number of other proteins. These include the DNA binding protein *ner* from phage Mu, human interleukin-1β and a trypsin inhibitor from *Ascaris*. In the case of human interleukin-1β, extensive use of 3D and 4D heteronuclear NMR has been made to resolve problems associated with spectral overlap and proton chemical shift degeneracy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29025-02-LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Investigations of Macromolecular Structures and Dynamics in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientists	LCP-NIDDK
Others:	Paul Driscoll	Visiting Fellow	LCP-NIDDK
	Julie Forman-Kay	Guest Researcher	LCP-NIDDK
	Bruce Grassberger	IRTA Fellow	LCP-NIDDK
	Claudia Jansen	Guest Researcher	LCP-NIDDK
	Jim Omichinski	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any) Robert Powers IRTA Fellow LCP-NIDDK

LCP/NIDDK (Ad Bax, Lewis Kay, Attila Szabo); Protein Expression Laboratory (Paul Wingfield, Stephen Stahl); NCI (Ettore Appella, Pat Becerra, Koji Matsushima, Sam Wilson, Alex Wlodawer)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of the overall research in this laboratory is centered on achieving as complete a description as possible for the structures of peptides, proteins, nucleic acids and their complexes in solution, principally by NMR spectroscopy. At present particular emphasis is being placed on developing approaches which allow the investigation of larger systems as well as increase the precision with which these solution structures can be obtained.

Structures for several proteins have been determined and analyzed. These include the Zinc-finger domain of a human enhancer binding protein, human thioredoxin, and the cytokine interleukin-8. These solution structures represent high resolution structures, with rmsd values of approximately 0.4 angstroms for the backbone and 0.6-0.9 angstroms for all atoms.

In addition to structural studies, novel NMR technology allows the detailed study of protein dynamics and a detailed investigation has been carried out for the protein interleukin-1 $\beta$ . Using two-dimensional inverse detected heteronuclear N-15/H-1 NMR spectroscopy, N-15 spin-lattice and spin-spin relaxation times and NOE data were collected and analyzed, demonstrating motions on three different time scales for the backbone amide groups.

Work is in progress on several other proteins, such as the DNA binding protein  $\nu$ er from phage Mu, several interleukins, the RNase-H domain of HIV-1 reverse transcriptase and Ascaris trypsin

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29026-02-LCP

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NMR and Other Spectroscopic Studies of Molecular Structure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Edwin D. Becker Research Chemist LCP-NIDDK

## COOPERATING UNITS (if any)

Sophisticated Instruments Facility, Indian Institute of Science, Bangalore, India; Centre for Cellular and Molecular Biology, Hyderabad, India (both foreign).

I. W. Levin, LCP, NIDDK

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

NMR Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

- A. Collaborative studies with C. L. Khetrpal and others at the Indian Institute of Science use nuclear magnetic resonance (NMR) methods to study molecules oriented in liquid crystals. Because the spectra rapidly become very complex with increasing numbers of interacting hydrogen atoms, only small or highly symmetric molecules have been studied. We are exploring a new method to use solid-state NMR spinning techniques to extend the range of applicability of the method.
- B. A new analysis of the fundamental vibrations of p-benzoquinone has been carried out, using recently acquired Raman spectroscopic data. Several confusing aspects of the assignment have now been explained satisfactorily.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29027-02-LCP

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Theoretical studies of dynamical processes in chemical physics and biophysics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Robert Zwanzig Research Chemist LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysics Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The general focus of research has been on rate processes in disordered systems. One investigation dealt with diffusion in a disordered medium, and in particular with the effects of time- and space-dependent fluctuations in the diffusion coefficient. This is potentially useful in understanding chemical kinetics in biochemical processes in cells. In a second investigation, a new treatment has been made of Berg and Purcell's theory of diffusion controlled ligand binding to receptors randomly distributed on the surface of a cell. Aside from improving on our understanding of their theory, the new treatment makes a modest improvement on its accuracy. In a third area, a major review has been written about theoretical methods that have been invented to treat kinetic processes where rate constants can fluctuate in time (dynamical disorder). Since there are many areas of chemical physics and biophysics in which dynamical disorder is important (e.g. protein dynamics or fluorescence depolarization), a unified treatment of the various methods that have been proposed is expected to be generally useful.



ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research of the Laboratory is directed towards the introduction of new concepts, techniques and agents for the elucidation of the molecular nature of mechanisms controlling cell functions. Specific focus is placed on i) Development of selective agonists/antagonists for receptors controlling cyclic nucleotide formation, phospholipid metabolism and ion channel function; ii) The relationship between ion transport, phospholipid turnover and cyclic nucleotide generation and the delineation of agents with specific effects on macromolecules involved in these systems. iii) The isolation and structure elucidation of biologically active natural products and definition of the basis of their activity. iv) Effects of agents on ion channels and the development of radioactive ligands for modulatory sites in such channels. v) The nature of enzymes involved in formation and inactivation of neurotransmitters, hormones, and other modulatory substances, in particular the enzymes, catechol-O-methyltransferase, monoamine oxidase, adenylate cyclase and phosphodiesterases. vi) the fundamental mechanisms by which drugs and environmental chemicals are transformed in the body with emphasis on oxidative metabolism by cytochrome P-450 systems to generate active oxide metabolites that interact with macromolecules such as DNA and are metabolized by further oxidation, by hydrolysis and by conjugation with glutathione.

Some of the milestones for the Laboratory are i) Introduction of the adenine-prenylation technique for study of cyclic AMP generation in intact cells; ii) The steroidal alkaloid batrachotoxin as a selective activator of sodium channels. iii) Histricnicotoxin as a noncompetitive blocker of acetylcholine receptor channels and potassium channels. iv) Pumiliotoxins as myotonic and cardiotonic alkaloids acting through sodium channels to elicit phosphoinositide turnover. v) N<sup>6</sup>-Substituted adenosines, 8-phenyl and 8-cyclohexylxanthines and other heterocycles as selective and potent adenosine receptor agonists and antagonists suitable as radioligands for binding studies and for definition of A<sub>1</sub> and A<sub>2</sub> classes of receptors. vi) Introduction of forskolin as a specific and widely useful activator of adenylate cyclase. vii) Fluoronorepinephrines and analogs as selective alpha and beta-adrenergic agonists. viii) Production of antibodies to catechol-O-methyl transferase and their use in studying localization of this key catechol-metabolizing enzyme. ix) Development of a cyclic quaternary amine, isorecolone methiodide, with high potency and selectivity for nicotinic receptors. x) Definition of a relationship between receptor-activation of phosphoinositide breakdown; protein kinase C activation, and altered responses of cyclic AMP-generating systems. xi) Introduction of maitoxin as a general activator for phosphoinositide breakdown. xiii) Discovery of the NIH shift of aryl substituents during P-450 catalyzed phenol formation and demonstration of arene oxides as intermediates. xiv) Demonstration of oxidation-hydrolysis pathways that convert stereoselectively polycyclic aromatic hydrocarbons to ultimate diol epoxides that react with DNA. xv) Discovery and formulation of the bay-region theory, which is predictive of the pathway for formation of reactive carcinogenic metabolites from polycyclic aromatic hydrocarbons. xvi) Development of optical assays for protease and reverse transcriptase of HIV-1.

The laboratory accomplishes its mission both through its own resources and through extensive collaborations with other laboratories both at NIH, at Universities, Museums, and other institutes and in drug and chemical companies. Such collaborations can involve sharing of expertise on syntheses, isolations, analyses and biological testing and field work to obtain sources of new natural products.

## SECTION ON PHARMACODYNAMICS

### Pharmacologically Active Compounds from Amphibians and Other Sources

A number of new histrionicotoxins, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, decahydroquinolines, 5,8-disubstituted indolizidines, 6,9-disubstituted quinolizidines and tricyclic alkaloids have been detected and characterized from extracts of dendrobatid frogs. Structures can be proposed based on gas chromatographic mass spectrometry (GS-MS) and gas chromatographic Fourier transform infrared spectroscopy (GC-FTIR) in combination with exchange reactions and microchemical conversions. Further analysis of one class of dendrobatid alkaloids, from the Panamanian poison frog Dendrobates pumilio, namely alkaloids 222 ( $C_{13}H_{22}N_2O$ ); 236 ( $C_{14}H_{24}N_2O$ ) and 252 ( $C_{14}H_{24}N_2O_2$ ), which were thought to be amidine in nature, has revealed the true structures to be an oxime, an O-methyloxime and a hydroxy O-methyloxime of a tricyclic pyrrolizidine related in structure to the millipede alkaloid polyzonimine. The structure of another trace alkaloid 208/210 ( $C_{11}H_{13}N_2Cl$ ) from the Ecuadorean poison-frog Epipedobates tricolor has now been elucidated by nmr analysis of an N-acetyl derivative. Biological evaluation of analgetic activity is in progress.

Alkaloids from skins of the toad Melanophryniscus stelzneri were characterized using GC-MS and GC-FTIR. These bufonid toads contained a variety of "dendrobatid alkaloids" and some alkaloids hitherto unknown in amphibians. Two populations of M. stelzneri from Argentina were compared. One contained chiefly indolizidines (two diastereomers of 3-butyl-5-propyl- and three of 3-butyl-5-methyl-indolizidine) and cis- and trans-fused 2,5-dipropyl-, cis-2-propyl-5-pent-4-enyl- and cis-2-propyl-5-pentyl-decahydroquinolines). Traces of quinolizidines and the lady bug alkaloid precoccinelline were also found. The second population contained some of the same indolizidines and decahydroquinolines as the first with additional new quinolizidines and indolizidines and minor amounts of pumiliotoxin (PTX) 251D. A subspecies (montevideensis) from Uruguay contained mainly PTX 251D and minor amounts of three new homopumiliotoxins. In addition, minor indolizidines, pyrrolizidines and quinolizidines, some of which were identical with insect alkaloids, were found.

Alkaloids from the skins of various species of Madagascan frogs of the genus Mantella (Family Ranidae) were characterized using GC-MS and GC-FTIR. These ranid frogs contain a variety of "dendrobatid alkaloids" and a new class of alkaloids, previously unknown in nature. Four populations of M. madagascariensis all had major amounts of a quinolizidine that appears to be [6,9-Z]6-(cis-2-penten-4-ynyl)-9-methylquinolizidine (M.W. 217) and varying amounts of the 9-ethyl homolog (M.W. 231) and a dihydro-9-ethyl homolog. Three of the populations had substantial amounts of a 13,14-dihydroalloPTX-B (M.W. 325). All populations had either a 13,14-dihydroPTX-A (M.W. 309) or PTX-A (M.W. 307). Two of the

populations had a new alkaloid, tentatively identified in the PTX-A class with a 6-(2',5'-dimethylhex-4'-en-6-ol) side chain. Minor alkaloids included 3,5- and 5,8-disubstituted indolizidines and new alkaloids that appear to be 6,9-disubstituted quinolizidines. Two other mantellid species, M. aurantiaca and an apparently undescribed species, did not contain the 217, 231 or 233 quinolizidines but instead an alkaloid ( $C_{11}H_{23}NO$ , M.W. 235) representing a new class of alkaloids of unknown structure having 2-double bonds, an acetylatable hydroxy group and a major mass spectral ion at  $m/z$  162 ( $C_{11}H_{16}N$ ). These species contained a number of known PTX alkaloids, namely 267C, PTX-A, PTX-B and alloPTX 323B, an unknown ketonic PTX (M.W. 305) and an unknown indolizidine (M.W. 295). A fourth species, M. viridis, contained mainly PTX-A and PTX-B along with minor amounts of the 217 quinolizidine and a ketonic PTX (M.W. 322). Many of these alkaloids have activity at nerve and muscle.

Biologically active substances in extracts from dried mucous secretions from the Amazonian tree frog Phyllomedusa bicolor have been isolated by HPLC. Substances that interact at opioid, muscarinic and  $A_1$ -adenosine receptors were detected. The substances that interact at  $A_1$ -receptors, if they are agonists, would account for the profound behavioral depression elicited in mice by these extracts. Further study centered on the substances interacting at adenosine receptors. Three fractions were obtained. Two were inhibitory to binding of radiolabelled agonists to  $A_1$  receptors, while the third stimulated binding. All were peptides and the stimulatory peptide (mol. wt. 2870, 30 amino acid residues) was sequenced by Edman degradation. Synthesis is in progress as is further biological evaluation.

Dendrobatid frogs reared in captivity do not produce alkaloids. Two possible mechanisms for lack of alkaloids are under investigation with captive-bred frogs. These are i) lack of environmental input necessary for expression of the genome responsible for biosynthetic enzymes and ii) lack of dietary precursors. A variety of stresses, hormone-treatments and environmental manipulations have not led to formation of alkaloids. Alkaloids can be accumulated into the skin from diet and are retained for several months. No further metabolism of such alkaloids occur. At present alkaloids have been produced in captive-raised frogs only when raised in outside terrariums with natural lighting on wild-caught fruit flies and termites. Further studies are in progress.

Structure activity relationships of 5,8-disubstituted indolizidines and decahydroquinolines as noncompetitive blockers at two classes of nicotinic receptors-channel complexes, namely the muscle-type of Torpedo electroplax and the ganglionic-type of pheochromocytoma cells have been determined. Similar profiles obtained at both classes.

## SECTION ON PHARMACODYNAMICS

### Pharmacology and Metabolism of Biogenic Amines and Related Compounds.

COMT. An improved technique for the final purification step of COMT has been developed utilizing isolation and elution from Immobilon-P. Eight COMT-specific peptides from trypsin hydrolysates of the 23kD form of soluble rat liver COMT have been isolated, sequenced, and used to derive cDNA probes. An improved polymerase chain reaction has been utilized to identify new clones specific to the COMT-specific peptides. A putative DNA sequence has been obtained for the rat liver enzyme.

Light microscopic immunocytochemical observations of the localization of COMT in rat uterus were made with a specific antibody to the soluble form of rat liver COMT and peroxidase conjugated with streptavidin. In the nonpregnant rat, COMT was minimal but detectable in the uterine luminal and glandular epithelium with greater amounts present in uteri from rats in estrus than diestrus. In early pregnancy a robust but transitory induction of COMT was observed in the luminal epithelium. To more precisely define both the timing and factors contributing to the transitory appearance of COMT, uteri were examined on days 1-5 in pregnant and pseudopregnant rats. The induction of COMT in the luminal epithelium was observed on day 3 and 4 in uteri from pregnant, pseudopregnant, delayed implantation and lactating post partum rats. However, no COMT was observed in uteri from non-lactating post partum rats. Ovariectomy on day 0 or 1 but not on day 2 of pregnancy prevented the appearance of COMT on 4. Progesterone-treatment immediately following ovariectomy on day 0 or 1 of pregnancy restored the induction of COMT. In addition to pregnancy, the present study suggests that the stimulation of copulation and lactation are factors contributing to the induction of COMT in the endometrial epithelium possibly mediated by progesterone.

Studies on the role of COMT in the possible biosynthesis of mammalian opioids have included the O-methylation of S- and R-norcoclaurine and norcoclaurine-1-carboxylic acid. Enzymatic O-methylation of norcoclaurine-1-carboxylic acid and the derived dihydroisoquinoline yielded exclusively the 7-O-methyl ethers. High stereoselectivity was also observed with (-)-S and (+)-R-norcoclaurine. O-Methylation of (-)-S-coclaurine afforded 80% as the 6-O-methyl ether and 20% of the 7-O-methyl ether while the reverse was observed with (+)-R-coclaurine. The observed regioselectivity suggests that isoquinoline-1-carboxylic acids are not likely intermediates in the biosynthesis of (S)-reticuline. The formation of 80% of the 6-O-methyl ether from (S)-norcoclaurine is similar to the O-methylation observed in plant species and suggests that the mammalian pathway for the synthesis of opioids may be similar. Preliminary studies have been carried out on the enzymatic O-methylation of the next step in the proposed biosynthetic pathway leading to the formation of (-)-S-reticuline. The putative intermediates, (-)-S-demethylnorreticuline and (+)-R-demethylnorreticuline, have been prepared, as well as the authentic products, the 3- and 4-O-methyl ethers, S-norreticuline and R-orientaline. Both forms proved to be substrates for COMT, however the (-)-S-demethylnorreticuline is a better substrate than the (+)-R-enantiomere. The predominant product from (-)-S-demethylnorreticuline appears to be the 4-O-methyl ether norreticuline.

Release of Catecholamines: PC12 cells are a nerve growth factor-responsive clone derived from a rat pheochromocytoma. The cells contain catecholamines and secrete them in response to depolarizing stimuli and cholinergic agonists. Treatment of the cells with nerve growth factor produces a number of very rapid changes including the structural rearrangement of cell membrane, the generation of a number of different second messengers, and the phosphorylation of several proteins. Nerve growth factor treatment increases the release of dopamine and norepinephrine from the cells within a few minutes and does so independently of its effect on their metabolism. This effect of nerve growth factor is dependent on the presence of extracellular calcium and can be blocked by calcium channel antagonists. K-252A, an inhibitor of protein kinases, usually found to inhibit the actions of nerve growth factor on PC12 cells, also increases the release of catecholamine.

Fluorine Derivatives of Biogenic Amines: The following difluoro derivatives have been investigated: 2,6-difluorophenylephrine, 2,5- and 2,6-difluoronorepinephrine and 2,5-

and 2,6-difluoronepinephrine. The  $\alpha_2$ -agonist potency of 2,5- and 2,6-difluoronepinephrine was assessed by measuring the inhibition of forskolin-stimulated adenylate cyclase activity in human platelet membrane preparations. The potency of the 2,5-derivative ( $IC_{50} = 68 \mu M$ ) and the 2,6-derivative ( $IC_{50} = 37 \mu M$ ) were intermediate between the potencies of the weak  $\alpha_2$ -adrenergic agonist 2-fluoronepinephrine ( $IC_{50} = 110 \mu M$ ) and the more potent  $\alpha_2$ -agonist 6-fluoronepinephrine ( $IC_{50} = 3 \mu M$ ). The affinity of 2,6-difluoronepinephrine for  $\alpha_2$ -adrenergic receptor binding sites, as measured by the displacement of the specific binding of [ $^3H$ ]clonidine in rat brain membranes, is relatively weak ( $K_d = 0.8 \mu M$ ) and similar to the low affinity for 2-fluoronepinephrine ( $0.7 \mu M$ ), whereas the affinity of 6-fluoronepinephrine ( $0.01 \mu M$ ) is slightly greater than that of the parent, norepinephrine ( $0.03 \mu M$ ). The present results suggest that the negative influence of a fluorine on the 2-carbon of 2,6-difluoronepinephrine with regard to activity at  $\alpha_2$ -adrenergic receptors seems to predominate over the  $\alpha$  selective influence of fluorine on the 6-carbon.

## SECTION ON PHARMACODYNAMICS

### Ion Channels, Receptors and Second Messengers in the Nervous System.

Maitotoxin. Effects on Insulin Release, Phospholipid Turnover and Calcium. Maitotoxin (MTX) a high molecular weight marine natural product is a unique agent for activation of phosphoinositide breakdown and calcium uptake in intact cells. In hamster insulinoma (HIT) cells, MTX induces a time-dependent and concentration-dependent release of insulin that requires the presence of extracellular calcium. The response is not inhibited by the L-type calcium channel blocker nifedipine, is partially inhibited by cadmium, and is nearly completely blocked by cinnarizine. MTX induces  $Ca^{2+}$  uptake in these cells in a dose-dependent mode, and the uptake is blocked with nifedipine, cadmium, and cinnarizine. MTX-induced phosphoinositide breakdown in HIT cells is not affected by nifedipine or cinnarizine and is only partially blocked by cadmium. The results suggest that MTX-elicited release of insulin is attained by two mechanisms: i) a nifedipine-sensitive action, which results from MTX-induced activation of L-type calcium channels. This can be mimicked with high potassium concentrations; and ii) a nifedipine-insensitive action, which may be initiated by the activation of phosphoinositide breakdown by MTX. Such an activation of phospholipase C would result in the formation of  $IP_3$ , subsequent release of intracellular calcium and then release of insulin to the extracellular space. Cinnarizine is proposed to block both mechanisms, the first by blockade of calcium channels and the second by blocking  $IP_3$ -induced release of internal calcium.

Local Anesthetics and Phospholipid Metabolism. Various local anesthetics enhance the incorporation of [ $^3H$ ]inositol into phosphoinositides in guinea pig cerebral cortical synaptosomes. Dibucaine, QX-572 and dimethisoquin showed maximum stimulation of 100  $\mu M$ , tetracaine and diphenhydramine at 300  $\mu M$ , and QX-314 at 1 mM, while quinacrine, lidocaine and cocaine showed no or only slight stimulation. There was no correlation between local anesthetic activity, estimated by inhibition of the  $^{22}Na^+$  flux elicited by the sodium channel activator batrachotoxin, and the potency for stimulation of inositol incorporation. Although dibucaine and QX-572 markedly stimulated incorporation of [ $^3H$ ]inositol into phosphoinositides, only QX-572 significantly enhanced the incorporation of [ $^{32}P$ ]phosphate into phosphoinositides. The result suggest that certain local anesthetics enhance a pathway involving an exchange reaction between inositol and the phosphoinositol ester bond of phosphatidylinositol, but do not markedly affect the de novo pathway of phosphoinositide synthesis.

Effects of Forskolin on Ion Channels in Pheochromocytoma Cells. Forskolin, a naturally occurring diterpene that activates adenylate cyclase, HL706, a water-soluble derivative of forskolin (6 $\beta$ -[piperidino]acetoxy]-7-desacetylforskolin) that is less potent than forskolin in activating adenylate cyclase, and 1,9-dideoxyforskolin, an analogue that does not activate adenylate cyclase, were examined for effects on the nicotinic receptor-mediated  $^{22}\text{Na}^+$  flux, a high potassium-induced  $^{45}\text{Ca}^{2+}$  flux through L-type calcium channels, a high and potassium-induced  $^{86}\text{Rb}^+$  efflux through a calcium-dependent potassium channels in PC12 cells. Forskolin and analogues at 30  $\mu\text{M}$  completely blocked carbamylcholine-elicited flux of  $^{22}\text{Na}^+$  through the nicotinic receptor-gated channel. 1,9-Dideoxyforskolin had an  $\text{IC}_{50}$  value of 1.6  $\mu\text{M}$  with forskolin and HL706 being two- to three fold less potent. Forskolin and its analogues appear to be noncompetitive blockers of the ginglyonic nicotinic receptor-channel complex in PC12 cells, but unlike many noncompetitive blockers, did not markedly enhance desensitization. Instead, forskolin, but not HL706 or 1,9-dideoxyforskolin, slightly antagonized the desensitization evoked by high concentrations of carbamylcholine. 1,9-Dideoxyforskolin at 30  $\mu\text{M}$ , but not forskolin or HL706, markedly inhibited depolarization-evoked  $^{45}\text{Ca}^{2+}$  flux and  $^{86}\text{Rb}^+$  efflux in PC12 cells, suggesting that 1,9-dideoxyforskolin has nonspecific inhibitory effects on a variety of ion channels.

Irreversible Local Anesthetics: Effects on Sodium Channels.  $^{22}\text{Na}^+$  influx and binding of [ $^3\text{H}$ ]batrachotoxinin-A 20- $\alpha$ -benzoate ([ $^3\text{H}$ ]BTX-B) were studied in guinea pig cerebral synaptoneuroosomes. BTX induced a dose-dependent stimulation of sodium influx in synaptoneuroosomes ( $\text{EC}_{50}$  280 nM). The potency of BTX for stimulation of sodium influx was increased ( $\text{EC}_{50}$  24 nM) in the presence of 0.6  $\mu\text{g/ml}$  scorpion venom without any change in maximal influx. In contrast, specific binding of [ $^3\text{H}$ ]BTX-B to synaptoneuroosomes was minimal in the absence of scorpion venom, but it was increased several fold in the presence of 60  $\mu\text{g/ml}$  scorpion venom. The inhibition of [ $^3\text{H}$ ]BTX-B-binding by proparacaine isothiocyanate (PROPRIT), an irreversible local anesthetic, did not occur in parallel with an inhibition of sodium influx induced by BTX. Thus, preincubations of synaptoneuroosomes with 100  $\mu\text{M}$  PROPRIT for 10 min completely inhibited [ $^3\text{H}$ ]BTX-B binding, and under these conditions BTX-induced sodium flux was reduced only by 50%. It is proposed that binding of [ $^3\text{H}$ ]BTX-B in the presence of high concentrations of scorpion venom detects sites associated with sodium channels in synaptoneuroosomes far in excess beyond those required to induce maximal influx of sodium by BTX.

## SECTION ON OXIDATION MECHANISMS

### Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites.

Racemic samples of quinoline 5,6-oxide and quinoline 7,8-oxide have been synthesized by two methods from the corresponding dihydroquinoline precursors. *Trans*-5,6-dihydroxy-5,6-dihydroquinoline and *trans*-7,8-dihydroxy-7,8-dihydroquinoline were obtained both by multistep synthetic routes from the corresponding dihydroquinolines and by the direct base catalyzed hydration of the corresponding arene oxides.

Optically pure enantiomers of the diastereomeric pair of benzo[g]chrysene-11,12-diol 13,14-epoxides were synthesized from (11R,12R)- and (11S,12S)-dihydroxy-11,12-dihydrobenzo[g]chrysenes. Both hydration of the tetrahydro 11,12-epoxide and  $\text{NaBH}_4$  reduction of benzo[g]chrysene-11,12-dione were employed

to synthesize the racemic dihydrodiol. Diastereomeric bis(-)-menthyloxy esters of the tetrahydrodiol are much more effectively separated by HPLC than are the corresponding derivatives of the dihydrodiol. The conformational preference of the hydroxyl groups in the series-1 diol epoxide in which the benzylic 11-hydroxyl group and the epoxide oxygen are *cis* was found to be dependent upon solvent, with a quasidiequatorial conformation being preferred in DMSO- $d_6$  and a quasidaxial conformation being preferred in  $CDCl_3$ . As anticipated from prior studies of the rates of hydrolysis of the 3,4-diol 1,2-epoxides of benzo[c]-phenanthrene, the present 11,12-diol 13,14-epoxides are relatively stable in neutral, aqueous media. Enantiomers of the K-region 9,10-oxide were prepared from the K-region bromohydrin, which was resolved as diastereomeric esters with (-)- $\alpha$ -methoxy- $\alpha$ -(trifluoromethyl)phenylacetic acid. After separation by HPLC, the bromo esters were treated with NaOMe to provide the enantiomers. NaOMe was found to open the arene oxide by preferential attack at C<sub>10</sub>, although C<sub>9</sub> is calculated to be the more reactive position.

Rates and products have been measured in 1:9 dioxane:water, ionic strength 0.1 M, at 25 °C for the solvolyses of the two diastereomeric bay-region 3,4-diol 1,2-epoxides derived from *trans*-3,4-dihydroxy-3,4-dihydrodibenz[c,h]acridine (diol epoxide-1, in which the epoxide and the benzylic hydroxyl groups are *cis* to each other, and diol epoxide-2, in which these groups are *trans*). Comparative study of these diol epoxides and their carbocyclic analogues, the dibenz[a,j]anthracene 3,4-diol 1,2-epoxides, showed that the nitrogen atom at position 14 in the ring had only a small influence (2- to 5-fold deceleration) on the rate constants for either acid-catalyzed ( $k_H$ ) or pH-independent ( $k_o$ ) solvolysis. In these diol epoxides, the nitrogen is not in direct conjugation with the presumed carbocationic intermediate formed at C-1. The use of a rapid-mixing technique permitted measurement of solvolysis rates for the dibenz[c,h]acridine diol epoxides at pH values down to ca. 1.2 ( $t_{1/2}$  200-500 ms). At these low pH values, a slight curvature in the plots of  $\log k_{obsd}$  vs pH was observed, consistent with only partial protonation of the nitrogen at the lowest pH values used. Dibenz[a,j]acridine 3,4-diol 1,2-epoxide-2, which has a nitrogen at position 7, undergoes hydronium ion catalyzed solvolysis ( $k_H$ ) about 23 times more slowly than does the corresponding carbocyclic dibenz[a,j]anthracene diol epoxide. For the dibenz[a,j]acridine 3,4-diol 1,2-epoxide, unlike the diol epoxides derived from dibenz[c,h]acridine, there is an unfavorable resonance form of the benzylic C-1 carbocation associated with solvolysis that places positive charge on nitrogen. At pH values <2, dibenz[a,j]acridine diol epoxide-2 exhibits reactivity consistent with a mechanism for solvolysis that involves protonation on oxygen of the already N-protonated substrate. This observation constitutes, to our knowledge, the first example of such a dicationic mechanism for solvolysis of a heterocyclic epoxide derivative of this type.

Chemical structures of the principal adducts formed from DNA upon reaction *in vitro* with the four optically active 3,4-diol 1,2-epoxides derived from the *trans*-3,4-dihydrodiol enantiomers of dibenz[a,j]anthracene have been elucidated at the nucleoside level. In addition to the structures of deoxyadenosine (dA) and deoxyguanosine (dG) adducts, complete chemical characterization of a deoxycytidine (dC) adduct thus formed is reported for the first time. The site of covalent attachment of the diol epoxide moiety to the nucleoside residue in all of these adducts is at the exocyclic amino group of the base as was deduced from chemical stability considerations and pH titration ( $pK_a = 2.1$  and  $9.3$  of a

dG adduct;  $pK_a = 2.2$  of a dA adduct and  $pK_a = 2.6$  of a dC adduct). The stereochemistry (cis or trans opening of the epoxide at the benzylic C-1 position) of each adduct was deduced on the basis of the  $^1H$  NMR spectrum of the corresponding pentaacetate ester. All the dibenz[a,j]anthracene adducts that have S-absolute configuration at the benzylic C-1 carbon of the tetrahydroaromatic moiety exhibit CD spectra with a positive band at 270-280 nm and a negative band at 290-300 nm. Thus, assignment of cis vs. trans addition for minor adducts whose NMR spectra were unobtainable due to their low level of formation could be made on the basis of their CD spectra and the known absolute configurations of the parent diol epoxides.

Dibenz[a,h]acridine can form two diastereomeric pairs of bay-region diol epoxides which are not positionally equivalent as a result of the nitrogen atom at position 7. We have assessed the structure-activity relationships resulting from heterocyclic nitrogen substitution by examining the mutagenic activity of these four bay-region diol epoxides in both bacterial and mammalian cells. In strains TA98 and TA100 of *Salmonella typhimurium*, the diastereomeric 10,11-diol-8,9-epoxides were 20 to 40 times more mutagenic than the corresponding 3,4-diol-1,2-epoxides. Furthermore, in strain TA100, dibenz[a,h]-acridine 10,11-dihydrodiol, the expected metabolic precursor of the 10,11-diol-8,9-epoxide, was metabolically activated by rat hepatic microsomes up to a 12-fold greater extent than the 3,4-dihydrodiol. In Chinese hamster V79 cells, the 10,11-diol-8,9-epoxide diastereomers were 20 to 80 times more mutagenic than their 3,4-diol-1,2-epoxide counterparts. Quantum mechanical calculations of the predicted ease of benzylic carbocation formation at C-1 and C-8 from the diol epoxides indicate that the 3,4-diol-1,2-epoxides should be less reactive due to resonance destabilization of the C-1 carbocation by the electronegative nitrogen atom. Decreased chemical reactivity of 3,4-diol-1,2-epoxides may explain their decreased mutagenic activity.

## SECTION ON OXIDATION MECHANISMS

### Mechanistic Enzymology of HIV Proteins.

Ac-Lys-Ala-Ser-Gln-Asn-Phe( $NO_2$ )-Pro-Val-Val-NH<sub>2</sub> (peptide I) and Thr-Phe-Gln-Ala-Phe( $NO_2$ )-Pro-Leu-Arg-Glu-Ala (peptide II) undergo hydrolysis between the p-nitrophenylalanyl and prolyl residues catalyzed by the proteases of HIV-1 and AMV, respectively. The specific hydrolyses of peptides I and II are accompanied by a decrease in their UV absorption at 269 nm ( $\Delta\epsilon = 1000$ ) and an increase at 316 nm ( $\Delta\epsilon = 600$ ). The use of microspectrophotometric cells allows continuous UV measurements on a volume (60 to 120  $\mu$ l) comparable to that required for the HPLC point assay previously in common use. At the highest substrate concentration possible under the assay conditions, neither enzyme is saturated with respect to its substrate; thus, good first-order kinetics were observed with both proteases, and values of  $V_{max}/K_m$  were obtained.

Continuous optical assays for DNA-polymerizing enzymes, based upon changes in the circular dichroism (CD) and ultraviolet (UV) spectra upon elongation of the double-helical portion of template/primer complexes,  $p(dA)_{40-60} \cdot p(dT)_{20}$  or poly(rA). $p(dT)_{12-18}$ , has been developed. DNA- and/or RNA-directed DNA polymerization catalyzed by the Klenow fragment of *E. coli* DNA polymerase I and reverse transcriptase from HIV-1 were monitored by following the increase in the



absolute magnitude of the (negative) circular dichroism band at 248 nm or the decrease in the UV absorption at 275 nm. These optical changes were linearly related to the extent of incorporation of dTMP into the template-primer as measured by a standard radiochemical assay. Determination, by means of these optical assays, of  $K_m$  and  $V_{max}$  for dTTP in the polymerization reaction catalyzed by the Klenow fragment gave values that were in close agreement with values measured radiochemically. These assays permit the continuous observation of the entire time course of polymerization with a single 60-120 microliter sample, and are thus especially advantageous for kinetics studies. Because of their simplicity and use of non-radioactive substrates, they are also well suited for the screening of inhibitors for these enzymes.

Significance for Biomedical Research and the Program of the Institute: To date this project has resulted in the development of more convenient assay methods for both RT and protease of HIV-1. Our eventual goal is elucidate the mechanisms of catalysis by, and assembly of, these enzymes (both of which are active as dimers) through the use of substrate analogs as well as of inhibitors for both catalysis and dimerization. The implications for rational design of anti-HIV drugs are clear. The development of rapid, continuous optical assays for reverse transcriptase and retroviral proteases is essential for mechanistic studies, and will also greatly facilitate the screening of potential inhibitors.

## SECTION ON OXIDATION MECHANISMS

### Mass Spectrometry of Drugs, Metabolites and Natural Products.

**Snake Pheromones:** In collaboration with LBC, NHLBI, snake pheromones are being investigated. A major effort involved an investigation of pheromone variation as a mechanism for speciation in garter snakes. The attractiveness pheromones of female garter snakes have been identified as a series of long-chain methyl ketones and behavioral studies have demonstrated that male snakes recognize and distinguish their own females by means of these pheromones. A second effort involved the Brown Tree snake from Guam. This snake is responsible for the total eradication and extinction of three species of birds on Guam. In addition, they are aggressive and bite humans. Children seem to be especially susceptible to the venom of these rear-fanged snakes. They have already cost the government over 25 million dollars in power outages as well. The methyl ketone fraction of these snakes has now been characterized. This involved the development of methods to locate the two unsaturations in the hydrocarbon chain. The advantage is that these methods may be used to locate unsaturations in other unknown compounds being studied in other projects. Biological testing of these compounds as an attractant has yet to be performed.

**Biologically Active Molecules from South Pacific Marine Organisms:** The collaboration on the structural investigation of these biologically active marine natural products has continued. These compounds are being investigated for their antiviral, antibacterial and anti-AIDS potencies. During the year, it has been extended to include the Natural Products Chemistry Section of PDRG, NCI (FCRC, Frederick, MD), and structures of several of their compounds have also been established through joint analyses.

**Natural Benzodiazepines in Brain and Plasma:** As part of an investigation by LNS, NIDDK into the presence and identity of natural benzodiazepines in body tissues and fluids, methods were developed to analyze these compounds at trace levels. Measurements are complete on the known benzodiazepines in brain and work is continuing on the unknown components and those present in plasma.

**Plasma Desorption Mass Spectrometry:** This instrument is particularly suited to the analysis of non-volatile and/or high molecular weight compounds and the demand for these analyses has greatly increased. The instrument has been enhanced with the installation of a reflectron to provide better resolution and its ionization mass capability is being increased by the incorporation of laser.

**Chinese Medicinal Compounds:** China has a long history of the use of medicinal plants for cures of various illnesses, but the active compounds in these are generally unknown. The composition of ten oils from various parts of five plant species have been determined.

**Cataloging of the Mass Spectra of Compounds:** In collaboration with NIST, spectra of compounds for which the structure has been elucidated at NIH are being obtained and added to the NIST mass spectra library data base. Spectra from both current and previously analyzed samples are being added to the library. The value of this data base to mass spectrometrists is immense. Over 100 compounds a month are being processed with the work supported by NIST.

**Sample Load:** During the past year, approximately 1700 samples were submitted for analysis.

## SECTION ON PHARMACODYNAMICS

### Adenosine Receptor Agonists and Antagonists

**Adenosine Agonist Activity:** The antinociceptive effects after intrathecal injection of six N<sup>6</sup>-substituted adenosine analogs and of 2-phenylaminoadenosine were compared with the known affinity for the A<sub>1</sub>- and A<sub>2</sub>-adenosine receptors. Adenosine analogs, substituted in the N<sup>6</sup>-position, had stereoselective structure-dependent antinociceptive effects in the tail flick and hot plate assays after intrathecal injection in mice. The antinociceptive activity or lack of activity for N<sup>6</sup>-R- and S-phenylisopropyl-adenosine (R- and S-PIA), N<sup>6</sup>-R- and S-2-phenylethyladenosine, N<sup>6</sup>-1,1-dimethyl-2-phenylethyladenosine (methylPIA), and N<sup>6</sup>-cyclooctyladenosine correlated with the affinity for central A<sub>1</sub>-adenosine receptors. An adenosine analog, 2-phenylaminoadenosine, selective for A<sub>2</sub>-adenosine receptors, was inactive. The results suggest that spinal A<sub>1</sub>-adenosine receptors are responsible for the antinociceptive effects of adenosine and its analogs after intrathecal injection.

Adenosine analogs were tested for their ability to relax carbamylcholine-contracted trachea *in vitro*. Based on EC<sub>50</sub> values, the rank order of potency was: 5'-N-ethylcarboxamidoadenosine (NECA) > 2'-chloroadenosine (2ClADO) > 5'-chloroadenosine (5ClADO) = N<sup>6</sup>-R-1-phenyl-2-propyladenosine (R-PIA) > N<sup>6</sup>-cyclohexyladenosine (CHA) > 2-phenylaminoadenosine (CV1808) > 5'-methylthioadenosine (MTA). The rank order of potency for NECA, 2ClADO and R-PIA is characteristic of an A<sub>2</sub>

subtype of adenosine receptor. 8-Para-sulphophenyltheophylline (8pST), a relatively nonselective adenosine receptor antagonist was used to antagonize tracheal relaxation elicited by adenosine analogs. 8pST antagonized the 2ClADO, CHA, R-PIA, and 5ClADO responses, but had little or no effect on the CV1808 and MTA responses. 8pST antagonized responses to NECA at concentrations of NECA up to  $\sim 30 \mu\text{M}$ , but had no effect on responses to higher concentrations of NECA. The differences in antagonist potency of 8pST and the clear biphasic response of NECA are indicative of two mechanisms of adenosine analog action leading to tracheal relaxation. One mechanism is mediated through a xanthine-sensitive site, at which NECA acted in a potent manner, while the other mechanism is insensitive to blockade by xanthines and is the major site of action of MTA and CV1808, as well as NECA at high concentrations. MTA is known to be an antagonist at  $A_2$ -adenosine receptors that stimulate adenylate cyclase activity, yet MTA did not antagonize the NECA-induced relaxation of trachea. Thus, the  $A_2$ -type adenosine receptors in smooth muscle appear different from the  $A_2$ -adenosine receptors that are linked to adenylate cyclase in other tissues.

The locomotor effects in mice of selective  $A_1$  and  $A_2$  adenosine agonists, antagonists, and combinations of agonists were investigated. The  $A_2$ -selective agonist APEC, an amine derivative of CGS21680, was a more potent locomotor depressant than its amide conjugates. The rank order of potency after intraperitoneal injection for adenosine agonists was NECA ( $\text{ED}_{50} 2 \mu\text{g/kg}$ ) > APEC ( $\text{ED}_{50} 16 \mu\text{g/kg}$ ) > CHA ( $\text{ED}_{50} 60 \mu\text{g/kg}$ ). The locomotor effects of NECA, APEC, and CHA were completely reversed by theophylline, but not by the peripherally active 8-p-sulphophenyltheophylline, indicating a central action of the adenosine agonists. An  $A_1$ -selective adenosine antagonist, CPT, ( $10 \text{ mg/kg}$ ) completely reversed the locomotor depressant effect of CHA ( $A_1$ -selective) and NECA (non-selective) at doses of agonists as high as twice the  $\text{ED}_{50}$  and shifted the dose response curves to the right, suggesting a primary involvement of  $A_1$  receptors. CPT did not affect the depressant effects of APEC at the  $\text{ED}_{50}$ , consistent with the  $A_2$ -selectivity of APEC. The depressant effects of APEC, but not of NECA or CHA, were reversed significantly by an  $A_2$ -selective adenosine receptor antagonist, CP-66,713. Low or sub-threshold doses of CHA potentiated the depressant effects of APEC. A sub-threshold dose of CHA did not alter the depressant effect of NECA, while a sub-threshold dose of APEC increased the depressant effects of low doses of CHA and NECA. Thus, it appears that  $A_1$  and  $A_2$  selective adenosine agonists have separate central depressant effects, which can be potentiative. The relatively high potency of NECA *in vitro* could be due to a synergism between an essential central  $A_1$  and a potentiative  $A_2$  receptor activation by this nonselective agonist.

**Adenosine Antagonists:** A series of derivatives of 7-deazapurines with varying substituents in the 2-, 6- and 9-position was synthesized in an attempt to improve the adenosine receptor affinity and  $A_1$  or  $A_2$  selectivity.<sup>3</sup> The adenosine receptor affinities were assessed by measuring the inhibition of [ $^3\text{H}$ ]R<sub>2</sub>phenylisopropyl-adenosine (R-PIA) binding to rat brain  $A_1$  and inhibition of [ $^3\text{H}$ ]5'-(N-ethylcarbox-amido)adenosine (NECA) binding to rat striatum  $A_2$  adenosine receptors. A selected set of compounds representing the main structural variations was further examined in adenosine receptor-coupled adenylate cyclase assays. The results indicate that 7-deazahypoxanthines have a potential for  $A_2$  selectivity, while all 7-deazaadenines are  $A_1$  selective. Introduction of a phenyl<sup>2</sup> residue in the 2-position of 7-deazaadenines increases  $A_1$  activity tremendously. 2-p-Chloro-phenyl-7,8-dimethyl-9-phenyl-7-deazaadenine is potent and specific for the  $A_1$  receptors of rat brain

( $K_i = 120$  nM), having no affinity for the  $A_2$  receptors of rat striatum. A 1-phenylethyl substituent at the 9-position was found to be superior to a phenyl residue in terms of  $A_1$  affinity. The most potent  $A_1$  antagonist in the present series is the highly  $A_1$  selective (790 fold) R-7,8-dimethyl-2-phenyl-9-(1-phenylethyl)-7-deazaadenine ( $K_i = 4.7$  nM), which is 30-35 times more potent at  $A_1$  receptors than its S-enantiomer.

A series of imidazo[4,5-e][1,4]diazepine-5,8-diones were synthesized from hypoxanthines. Certain of these cyclic homologs of caffeine, theophylline, theobromine, 3-isobutyl-1-methylxanthine and enprofylline were inhibitors of binding of adenosine analogous to rat brain  $A_1$  and  $A_2$ -adenosine receptors and were antagonists of  $A_2$ -adenosine receptors stimulatory to adenylate cyclase in rat PC12 cell membranes. Activity at adenosine receptors was lower than the corresponding xanthines, perhaps because imidazodiazepinediones contain a boat-shaped seven-membered ring rather than the planar heteroaryl ring system of the xanthines. The imidazodiazepinediones had low affinity for brain benzodiazepine sites.

## SECTION ON PHARMACODYNAMICS

### Interaction Between Second Messenger Systems.

Activation of protein kinase C (PKC) in intact cells can induce significant changes, either facilitatory or inhibitory on cyclic AMP accumulation, elicited either by receptor activation or by the activator of adenylate cyclase, forskolin. Such interaction represents an example of "cross-talk" between second messenger systems and may underlie the biochemical basis of synchronization between external stimuli and biological responses. PKC is now known to comprise a variety of subspecies. Although differences among the PKC subspecies are apparent in terms of their enzymological properties, no functional differences among them has been described. In PC12 cells where both  $\alpha$  and  $\gamma$  isozymes of PKC are present, activation of PKC has been found to cause enhancement of responses of cyclic AMP-generating systems. In NCB20 cells and NIH 3T3 cells, where only the  $\alpha$  isozyme is expressed, activation of PKC has been found to cause inhibition of cyclic AMP-generating systems. In NIH 3T3 cells, after transfection of the  $\gamma$ -PKC, activation of the enzyme was no longer inhibitory, but instead a facilitation of cyclic AMP accumulation was observed. Thus, the  $\alpha$  and  $\gamma$  isozymes of PKC appear to have opposite actions, facilitatory for  $\gamma$ -PKC, and inhibitory for  $\alpha$ -PKC, on responses of cyclic AMP-generating systems in NIH 3T3 cells. Such opposing actions represent a remarkable functional distinction between two PKC subspecies.

Maitotoxin (MTX) activates calcium channels and stimulates phosphoinositide breakdown in pheochromocytoma PC12 cells, while having no effect on basal levels of the cyclic nucleotides cAMP and cGMP. Atrial natriuretic factor (ANF) induces a dose-dependent accumulation of cGMP in PC12 cells through the activation of a membrane-bound guanylate cyclase. Effects of ANF on cGMP are independent of extracellular concentrations of calcium. Since agents that activate phosphoinositide breakdown can indirectly affect cyclic nucleotide formation, the effects of MTX on ANF-mediated accumulation of cGMP was studied. MTX induces a dose-dependent inhibition of ANF-mediated accumulation of cGMP. The inhibition by MTX requires the presence of extracellular calcium, but is unaffected by the calcium channel blocker

nifedipine. The inhibitory effect of MTX is not mimicked by the calcium ionophore ionomycin. A phorbol ester, PMA, which stimulates protein kinase C, also inhibits ANF-mediated accumulation of cGMP. Sodium nitroprusside induces large accumulations of cGMP in PC12 cells through the stimulation of a soluble guanylate cyclase. Neither MTX nor PMA inhibit nitroprusside-mediated accumulation of cGMP. The results indicate that in PC12 cells, protein kinase C activation, either directly with PMA, and indirectly with MTX through phosphoinositide breakdown and formation of diacylglycerol, leads to inhibition of ANF-mediated, but not nitroprusside-mediated accumulation of cGMP.

PKC activation results in inhibition of receptor-mediated phospholipase C activation in cells. In NIH 3T3 cells, a receptor for the peptide endothelin is coupled to phospholipase C. The response to endothelin is only marginally inhibited in cells pretreated with phorbol esters activators of PKC. In cells transfected with  $\gamma$ -PKC, pretreatment with phorbol esters resulted in almost complete blockade of endothelin-mediated phospholipase C activation. The results indicate an involvement of  $\gamma$ -PKC on desensitization of the phosphoinositide breakdown response to endothelin receptor. It was shown that in  $\gamma$ -PKC transfected cells upon stimulation with phorbol esters, a phosphorylation of the  $\gamma$ -isozyme of phospholipase C is observed. Such an effect is not observed in non-transfected cells, and suggest that phosphorylation of the  $\gamma$ -isozyme may explain the desensitization phenomenon observed in the presence of  $\gamma$ -PKC.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31100-25 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	C.R. Creveling	Research Chemist	LBC, NIDDK
	T. Spande	Research Chemist	LBC, NIDDK
	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK
	H.M. Garaffo	Visiting Fellow	LBC, NIDDK
	Y-L. Hu	Guest Worker	LBC, NIDDK
	J. Caceres	Chemist	LBC, NIDDK
	S. Secunda	Biologist	LBC, NIDDK

COOPERATING UNITS (if any) T. Tokuyama, Osaka City U., Japan; Y. Kanaoka, Hokkaido Univ., Sapporo, Japan; V. Erspamer, U. Roma; C.W. Myers, Am. Mus. Nat. History, NYC; Balt., MD; R.S. Aronstam, U. GA., Augusta, GA; E. Gros, Univ. Buenos Aires, Argentina, J. Cover, A. Wisniewski, Nat. Aq. Balt., MD; C. Nishihira, Honolulu, HI.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.3

## PROFESSIONAL:

3.3

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Natural products have provided a wide range of biologically active agents, many of which have unique profiles of pharmacological activity and therapeutic potential. Over two hundred alkaloids have been identified in extracts from amphibian skins. These include batrachotoxins, which are potent activators of sodium channels, histrionicotoxins, which are noncompetitive blockers of nicotinic receptor channel complexes and of potassium channels, and pumiliotoxins, which have myotonic and cardiotoxic activity due to inhibitory effects on closing of sodium channels. A variety of further alkaloids have been characterized from dendrobatid frogs. These include new 2,5-disubstituted decahydroquinolines 5,8-disubstituted indolizidines, 6,9-disubstituted quinolizidines, 3,5-disubstituted pyrrolizidines, pumiliotoxins, homopumiliotoxins and allo-pumiliotoxins. Many of these alkaloids have activity as noncompetitive blockers at muscle-type and ganglionic-type nicotinic receptor-channels. Structures for three tricyclic pyrrolizidinone oximes and O-methylloximes from a dendrobatid frog have been determined based on analysis by mass spectrometry, infrared spectroscopy and nuclear magnetic resonance spectrometry. A structure for the parent member ( $C_{11}H_{13}N_2Cl_2$ ) a new class of analgetic alkaloids has been similarly derived by analysis of an N-acetyl derivative. Characterization of alkaloids in nondendrobatid amphibians indicates that the biosynthetic pathways to certain dendrobatid alkaloids have evolved separately in one lineage (genus) of amphibians from the families Bufonidae, Myobatrachidae and Ranidae and in a lineage leading to four genera in the family Dendrobatidae. The lack of alkaloid production in captive-raised dendrobatid frogs now appears due to lack of natural environment and/or diet. A peptide (thirty amino acid residues) isolated from mucous secretions of a hyliid frog Phyllomedusa bicolor causes profound behavioral depression and stimulates binding of adenosine agonists to brain  $A_1$ -adenosine receptors.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31101-22 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacology and Metabolism of Biogenic Amines and Related Compounds

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	C.R. Creveling	Research Chemist	LBC, NIDDK
Others:	J.W. Daly	Chief	LBC, NIDDK
	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK

## COOPERATING UNITS (if any)

Kirk, LC, NIDDK; Brossi, LAC, NIDDK; Guroff, GF, NICH; Seaman, NCDB, FDA; Grossman, U. Penn., Phil. PA; Inoue, Okayama U., Okayama, Japan.; Thakker, Glaxo Inc., Res. Triangle, NC.; Vesser, U. Ulm, Ulm, GDR; Nagatsu, U. Nagoya, Japan.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

1.0

## PROFESSIONAL:

1.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The biochemistry, physiology, and pharmacology of biogenic amines, amino acid precursors and metabolic products, and various synthetic derivatives have been investigated. The general areas of study include the effects of fluorine substitution on the properties of biogenic amines, adrenergic antagonists and amino acids and catechol-O-methyltransferase (COMT). Studies specific to fluorine-substituted compounds include 1) determination of the adrenergic properties of 2- and 6-fluoro derivatives of dopamine, epinephrine, 3-*t*-butyl-amino-1-(3,4-dihydroxyphenoxy)-2-propanol and fluoro- $\alpha$ -prenolols and fluoro-practolols 2) determination of adrenergic properties of 2- and 6-chloro and 2- and 6-methyl derivatives of norepinephrine 3) mechanism of toxicity of 6-fluoro- and 2,6-difluorophenylalanine and 6-fluoro-, 2,3-, 2,5-, 3,5-, and 2,6-difluoro-tyrosine in cultured pheochromocytoma (PC12) cell lines. The effect of nerve growth factor on release of catechol amines from pheochromocytoma PC12 cells has been investigated. Studies specific to COMT include: 1) immunohistochemical localization of COMT in the luminal and glandular epithelium of at uterus during estrus, diestrus, pregnancy, pseudopregnancy, postpartum and following ovariectomy in animals treated with and without exogenous progesterone 2) the immunofluorescent localization of COMT in the rodent and human intestinal tract 3) substrate specificity and reaction kinetics of a series of isoquinolines and tetrahydroisoquinolines as putative precursors of mammalian opioids 4) the primary sequence of peptides isolated from the 23KD form of a rat liver soluble COMT, preparation of a cDNA for COMT. 5) Determination of the number of genes coding for COMT and the chromosomal location and arrangement and transcriptional control of their expression.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31102-19 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ion Channels, Receptors and Second Messengers in the Nervous System

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK
	C.R. Creveling	Research Chemist	LBC, NIDDK
	O. Choi	Visiting Fellow	LBC, NIDDK
	M. Murata	Visiting Fellow	LBC, NIDDK
	P. Holbrook	NRC Fellow	LBC, NIDDK
	W. Padgett	Biologist	LBC, NIDDK
	D. Soergel	Biologist	LBC, NIDDK

## COOPERATING UNITS (if any)

T. Yasumoto, Tohoku Univ. Sendai, Japan, K. Seamon, NCDB, FDA, R.A. Aronstam, Univ. GA, Augusta, GA.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.2

## PROFESSIONAL:

3.7

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
 ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Calcium, sodium, potassium, and magnesium, ions can serve after translocation through ion channels or by transport proteins as second messengers to cause activation of release processes, contractile proteins, adenylate and guanylate cyclase, phosphodiesterases, protein kinases, phospholipases, ATPases and other enzymes. Receptors of various types and various toxins serve to modulate ion channels and generation of second messengers. Maitotoxin (MTX) activates calcium uptake and phosphoinositide breakdown in a wide range of cells. The two responses appear to be independent actions of MTX in that MTX-elicited calcium uptake can be greatly inhibited with no effect on MTX-elicited phosphoinositide breakdown. Lower concentrations of MTX (30 pM) are required to elicit phosphoinositide breakdown than are required to elicit significant calcium uptake. A radiolabelled MTX has been prepared and is being investigated in binding studies. In insulinoma cells MTX-elicited release of insulin appears due to release of calcium from internal stores as a result of phosphoinositide breakdown and generation of inositol trisphosphate ( $IP_3$ ). MTX-elicited influx of calcium in insulinoma cells appears nonessential to release since it can be blocked by nifedipine without blocking release. Remarkably, cinnarizine blocks calcium influx and release; it appears to block release by blocking  $IP_3$ -induced release of internal calcium. Local anesthetics enhance incorporation of radioactive inositol into phospholipids apparently by enhancing an exchange pathway. Forskolin and analogs are noncompetitive blockers of ganglionic nicotinic receptor channel complexes in pheochromocytoma cells, but unlike other non-competitive blockers do not enhance desensitization. An irreversible local anesthetic, proparacaine isothiocyanate, inhibits binding of a radioactive batrachotoxin to sodium channels to a much greater extent than inhibition of batrachotoxin-elicited sodium flux, indicating a large excess of sodium channels in synaptoneurosome beyond those needed to induce a maximal influx of sodium.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31104-22 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: D.M. Jerina Section Chief LBC, NIDDK

Others: J.M. Sayer	Research Chemist	LBC, NIDDK
H. Yagi	Visiting Scientist	LBC, NIDDK
A.M. Cheh	Research Chemist	LBC, NIDDK
D.R. Bushman	Staff Fellow	LBC, NIDDK
N.T. Nashed	Special Expert	LBC, NIDDK
A. Chadha	Visiting Fellow	LBC, NIDDK

COOPERATING UNITS (if any) A. Conney, Rutgers U. (Newark, NJ); W. Levin and A. Wood, Roche Research Center (Nutley, NJ); D. Whalen, Univ. of MD (Catonsville); D. Boyd, Dept. of Chem., Queen's Univ. of Belfast (N. Ireland); R. Lehr, Univ. of Oklahoma (Norman, OK); G. Holder, Univ. of Sydney (Australia)

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Oxidation Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6

## PROFESSIONAL:

5

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The primary goal has been the elucidation of the structures of reactive metabolites responsible for the carcinogenic, cytotoxic and mutagenic activity of drugs, polycyclic aromatic hydrocarbons, and other environmental chemicals. The approach taken consists of: i) synthesis of primary and secondary oxidative metabolites, ii) study of the metabolism of the chemicals with liver microsomes, as well as with purified and reconstituted cytochrome P-450 systems with and without epoxide hydrolase, iii) evaluation of the mutagenicity and tumorigenicity of the synthetic metabolites, iv) elucidation of the roles of the cytochrome P-450 system and epoxide hydrolase in modulating the mutagenicity of these metabolites, v) determination of the rates and products of reactions of arene oxides and diol epoxides with biopolymers and model compounds, and vi) search for agents capable of preventing the tumorigenicity of reactive metabolites. Synthetic studies have now made available optically pure bay-region 11,12-diol-13,14-epoxides of benzo[*g*]chrysene for testing of their mutagenic and tumorigenic activities. Comparative solvolytic studies of the bay-region 3,4-diol-1,2-epoxides of dibenz[*a,j*]anthracene with aza-analogs in which nitrogen has been substituted for CH at positions 7 or 14 have shown that nitrogen at position 14 has little effect (2 to 5-fold deceleration) on solvolytic reactivity in either the acid catalyzed or pH-independent (neutral to basic) regions whereas nitrogen at position 7 markedly decreases (23-fold) the acid-catalyzed reaction. A novel dicationic acid-catalyzed reaction mechanism has been observed in this latter case. Examination of the covalent bonding of the optically active bay-region-3,4-diol 1,2-epoxides of dibenz[*a,j*]anthracene to calf thymus DNA in vitro has allowed identification of a novel cytosine adduct in addition to the anticipated 16 adducts at adenine and guanine. Mutagenicity studies in bacteria and in Chinese hamster V79 cells have shown the 10,11-diol 8,9-epoxides of dibenz[*a,h*]acridine are more potent mutagens than the 3,4-diol 1,2-epoxides in accord with their differences in chemical reactivity.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31105-05 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Nicotinic and Muscarinic Acetylcholine Receptor Agonists.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: J.A. Waters

Research Chemist

LBC, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided.)

Terminated

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK31106-03 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanistic Enzymology of HIV Proteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	D.M. Jerina	Section Chief	LBC, NIDDK
Others:	J.M. Sayer	Research Chemist	LBC, NIDDK
	N.T. Nashed	Special Expert	LBC, NIDDK
	B.J.R. Forbes	Staff Fellow	LBC, NIDDK
	J.G. Baillon	Visiting Fellow	LBC, NIDDK

## COOPERATING UNITS (if any)

J. M. Louis, LCDB, NIDDK; P. T. Mora, OD, DCBD, NCI; S. Oroszlan, NCI-FCRDC, Frederick, MD

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Oxidation Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.6

## PROFESSIONAL:

2.5

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Methods of enzymology, chemical and enzymatic kinetics, and synthetic, physical and analytical chemistry are being used to investigate the mechanisms of action of reverse transcriptase (RT) and protease enzymes of HIV-1, with the ultimate goal of developing specific inhibitors for these enzymes. To facilitate these studies, optical assays for retroviral proteases and RTs have been developed. i) Cleavage of the chromogenic substrates, Ac-Lys-Ala-Ser-Gln-Asn-(p-nitro)Phe-Pro-Val-Val-amide and Thr-Phe-Gln-Ala-(p-nitro)Phe-Pro-Leu-Arg-Glu-Ala, catalyzed by the retroviral proteases of HIV-1 and avian myeloblastosis virus (AMV), respectively, occurs specifically between the p-nitrophenylalanyl and prolyl residues. These hydrolyses are accompanied by an increase in UV absorption at 316 nm, which permits convenient, continuous monitoring of the progress of these reactions. ii) Optical assays for RT and other DNA polymerases have been developed. These assays are based upon changes in circular dichroism (CD) and UV spectra upon elongation of the double helical portion of synthetic template/primer duplexes. In the presence of the Klenow fragment of *E. coli* DNA polymerase I or HIV-1 RT, incorporation of dTMP into a synthetic template-primer consisting of a 40-60-mer of dA, primed with a 20-mer of dT, produced an enhancement in the negative CD band at 248 nm and a decrease in the UV absorption at 260-275 nm. Both changes are linearly related to the extent of dTMP incorporation. Analogous results were obtained with HIV-1 RT using poly(rA) primed with oligo(dT). These continuous assay methods for retroviral enzymes offer a distinct advantage over single-point assays, in that they permit measurement of the entire time course of the enzymatic reaction using a single 60-120 microliter sample. These methods are presently being employed in studies of the effects of reaction conditions and specific functional-group reagents on the activity of both RT and HIV-1 protease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 31107-03 LBC

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mass Spectrometry of Drugs, Metabolites and Natural Products.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	L. K. Pannell	Visiting Scientist	LBC, NIDDK
Others:	Y. Murata	Special Volunteer	LBC, NIDDK
	Q-l. Pu	Special Volunteer	LBC, NHLBI
	D. M. Jerina	Section Chief	LBC, NIDDK
	J. W. Daly	Laboratory Chief	LBC, NIDDK

COOPERATING UNITS (if any) Becker, Whittaker and White, LAC, NIDDK; Basile, LNS, NIDDK; Fales, Mason, LBC, NHLBI, NIH; Munro and Blunt, U. Canterbury, New Zealand; Guangxi Inst. Trad. Med. Pharm. Sci.; Chem. Dept., Tohoku U., Sendai, Japan; M. Boyd, NCI, FCRC; S. Stein, Natl. Inst. of Stand. and Tech.; D.X. West, U. IL, Normal, IL.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Specialized mass spectrometry analyses are provided to the laboratory and to other collaborating units. The emphasis is primarily, but not exclusively, devoted to trace organic compounds isolated from biological systems. During the past year, a new high resolution mass spectrometer has been ordered and is currently being installed. This instrument will extend the capabilities of the section to analyze high mass compounds and to obtain accurate mass analyses on compounds evaporated from a probe or introduced by gas chromatography. Structural changes were made to the mass spectrometry facilities in order to house this new instrument within existing space. The Californium plasma desorption mass spectrometer is currently being fitted with a laser to extend the analysis of high molecular weight compounds. Major projects have involved the examination of attractants and repellents in snake skins, the objective being to use these to differentiate species or control snake habitat/population; the identification of natural benzodiazepines obtained from brain and their change in level as a function of certain illnesses; and the identification of biologically active natural products. Samples analyzed derive from many facilities and researchers outside LBC.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31108-02 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenosine Receptor Agonists and Antagonists

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	L.E. Brackett	IRTA Fellow	LBC, NIDDK
	O. Nikodijevic	Guest Worker	LBC, NIDDK
	C. Mueller	Guest Worker	LBC, NIDDK
	I. Hide	Guest Worker	LBC, NIDDK
	W. Padgett	Biologist	LBC, NIDDK
	S. Secunda	Biologist	LBC, NIDDK

COOPERATING UNITS (if any) C. Post, Astra, Sodertalje, Sweden; S.M. Anderson, Walter Reed Inst., Wash. D.C.; P.K. Bridson, Memphis State Univ., TN; M. Beavan, LC, NHLI; R. Sarges, Pfizer, Groton, CT; K. Jacobson, LC, NIDDK; R. Olsson, U. So. Fla., Tampa, FL; K. Eger, U. Tubingen, Germany, L. Gustaffson, Karolinska Inst., Sweden.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.1

## PROFESSIONAL:

3.1

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Adenosine regulates a wide range of physiological functions through interaction with at least two major classes of adenosine receptors. The  $A_1$  class of adenosine receptors is inhibitory to adenylate cyclase, while the  $A_2$  class is stimulatory to adenylate cyclase. Subclasses of adenosine receptors also occur. Some of these are inhibitory to calcium channels, some are stimulatory to potassium channels, some can activate guanylate cyclase, some can modulate phospholipid, while those of smooth muscle cause relaxation through a poorly defined mechanism. In the central nervous system activation of adenosine receptors cause behavioral depression, while blockade of adenosine receptors cause excitation. Antinociceptive activity of six N<sup>6</sup>-substituted adenosine correlated with  $A_1$  receptor potency. Adenosine analogs relaxed carbamylcholine-contracted trachea via two receptor mechanisms, one mechanism involved a xanthine-sensitive  $A_2$  receptor and the other a xanthine-insensitive receptor. The latter was the major site of action for 2-phenylaminoadenosine and 5'-methylthioadenosine. Adenosine analogs elicited central depressant effects via both  $A_1$  and  $A_2$  receptors. Potentative interactions between the two receptor systems appear to account for the high potency of nonselective agents, such as N-ethylcarboxamidoadenosine. A series of 7-deazapurines with substituents in the 2-, 6-, and 9-positions were synthesized. The most potent  $A_1$  antagonist was R-7,8-dimethyl-9-phenyl-9-(1-phenylethyl)-7-deazaadenine, which was 30-35 times more potent than its S-enantiomer. 2-p-Chlorophenyl-7,8-dimethyl-9-phenyl-7-deazaadenine was a potent and specific antagonist for brain  $A_1$  receptors. Imidazo[4,5-e][1,4]diazepine-5,8-diones, designed as cyclic homologs of caffeine, theophylline and other xanthines, were less active at adenosine receptors, than the corresponding xanthines, presumably because of the nonplanar ring system of the imidazodiazepindiones.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31109-01 LBC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Interaction between Second Messengers Systems.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK
Others:	J. Daly	Chief	LBC, NIDDK
	D. Soergel	Biologist	LBC, NIDDK
	A. Schulick	Guest Worker	LBC, NIDDK

COOPERATING UNITS (if any) J.S. Gutkind, LCDO, NIDR; A. Weissman, EIB, NCI; S.G. Rhee, LB, NHLBI; J. Bitran, W.Z. Potter, NSB, NIMH; J. Gardner, DD, NIDDK; R. Trullas, P. Skolnick, LN, NIDDK, B. Warren, MD Anderson, Texas.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.6

## PROFESSIONAL:

0.6

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

The activation of protein kinase C (PKC) either by phorbol esters or through breakdown of phospholipids and generation of the intracellular second messenger diacylglycerol, can modulate the formation of cyclic AMP (cAMP) and cyclic GMP (cGMP) induced by receptor agonists or direct activators of the cyclases. In terms of cAMP, such modulation mediated by PKC, can result in potentiation or inhibition of cAMP formation depending on the cell studied. A correlation was found between cells that express the  $\gamma$  isozyme of PKC and cells that exhibit potentiation of cAMP formation in response to PKC activation. NIH 3T3 fibroblasts normally do not express  $\gamma$ -PKC, and the formation of cAMP is inhibited in response to PKC activation. After transfection with  $\gamma$ -PKC-containing expression vectors, the fibroblasts now exhibit potentiation of cAMP formation after PKC activation by phorbol esters. PC12 rat pheochromocytoma cells contain a receptor for atrial natriuretic factor (ANF) that upon agonist binding stimulates a particulate guanylate cyclase activity. In addition, a soluble guanylate cyclase is present, which can be activated with compounds such as sodium nitroprusside. Activation of PKC in PC12 cells either with phorbol esters or through the breakdown of phosphoinositides and generation of diacylglycerol, results in inhibition of ANF-mediated, but not nitroprusside-mediated, guanylate cyclase activity. PKC isozymes affect differentially endothelin receptor-mediated activation of phospholipase C in NIH 3T3 fibroblasts. In the presence of  $\gamma$ -PKC an almost complete inhibition of endothelin-mediated stimulation of phospholipase C is observed, whereas in wild type cells only a slight inhibition (10%) occurs. In  $\gamma$ -PKC-transfected, but not in wild type NIH 3T3 cells, phosphorylation of phospholipase C ( $\gamma$ -isozyme) is observed. Such phosphorylation may explain the down regulation of endothelin-stimulated phosphoinositide breakdown in  $\gamma$ -PKC transfected cells.

## Annual Report of the Laboratory of Molecular Biology

### National Institute of Diabetes and Digestive and Kidney Diseases

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research program involves application of theoretical and experimental methods to a wide variety of problems in molecular genetics, regulation of gene expression in eukaryotes, mechanisms of DNA replication, nucleic acid and protein structure, bioenergetics and transport properties of biological molecules. These include studies of enzyme and immunoglobulin structure by X-ray diffraction, investigations of polynucleotide chemistry, structure and interactions by spectroscopic methods, studies by calorimetry of proteins and nucleic acids, studies on the molecular mechanisms for establishing and maintaining stable states of gene expression during embryogenesis, studies of the organization of DNA and proteins within the eukaryotic nucleus, studies of the effects of supercoiling on biological activity and protein-DNA interaction, as well as theoretical analyses of mechanisms of microtubule assembly, inter membrane diffusion of lipids and muscle contraction. There is increased interest in more direct studies of biological processes. These investigations include studies of the rearrangements that lead in vivo to the formation of assembled immunoglobulin genes, of the process of DNA replication in both prokaryote and eukaryotes, of the regulatory proteins that control expression of certain eukaryotic genes, of the effect of molecular crowding in biochemical systems, of non heritable antibiotic resistance and of the mechanism of genetic recombination. Significant progress has been made in all of these areas during the past year.

#### Structure of Enzymes and of Proteins of the Immune System

The three dimensional structure for McPC 603 Fab, which has been the basis for many structure determinations by molecular replacement, has been re-examined and refined using algorithms that have become recently available. The resulting structure, which is only a little different from the previously refined structure, shows a big improvement in stereochemistry. The structure of the McPC 603 Fab bound to phosphocholine has also been refined.

The crystal structure of the lactose specific lectin from the plant *Erythrina corallodendron* has been determined. A striking feature of the structure is the well ordered branched carbohydrate.

The crystal structure of the bifunctional enzyme complex, tryptophan synthase, has been refined at 2.1 Å resolution. This refined structure can now be used as a basis for examining changes that may accompany catalysis. In particular it appears that some of the residues

lining the "tunnel" that links the two active sites are not fixed rigidly in position, but may have a dynamic role.

Studies on two inhibitor complexes of the rhizopuspepsin have been directed towards a better understanding of the mechanism of action. The first, CP-69,799 was studied because previous work with endothiapepsin had suggested that it could produce a conformational change in the enzyme. No such change was observed with rhizopuspepsin. The second inhibitor provides a tetrahedral intermediate analog and permits better assignment of the substrate and enzyme protons.

Endonexin II, a calcium- and phospholipid-binding protein isolated from human placenta, has been successfully crystallized. X-ray diffraction data have been collected from native and heavy-atom treated crystals, and are currently being analyzed.

### Chromatin Structure and Function

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have continued to study the role played by the general erythroid-specific factor which we have named Eryf1. The cDNA for Eryf1 has been cloned, and we have studied the ability of the protein to serve as a transactivating factor in a variety of cells. The fact that very high levels of transcriptional stimulation are observed in certain cell types provides important information about the mechanism of activation by Eryf1. We have also cloned the cDNA for the human Eryf1. Comparison of the structure and activity of chicken and human proteins provides information both about their evolution and function. To learn more about factors that may act directly on chromatin structure, we have characterized in greater detail the binding of the factor BGPI, which binds to a string of 16 G residues in the adult  $\beta$ -globin hypersensitive domain. We have also surveyed chromatin of the entire  $\beta$ -globin domain for nuclease hypersensitive sites. This revealed four candidates for roles as dominant control regions (DCRs). Subsequent experiments in transgenic mice reveal that one of these, the  $\beta$ -globin enhancer already characterized in our laboratory, has the properties of a DCR. This provides an important potential connection between classical regulatory elements and chromatin structure.

### Control of Gene Expression during Chicken Erythrocyte Development

Developmental regulation of expression of two major gene families was studied: globin genes and HMG 14(a and b) and 17 genes. The former represents a differentiated function restricted to erythrocytes whereas the latter genes are constitutively expressed as an essential component of cellular chromatin.

Globin genes. Expression of the  $\epsilon$  globin gene is restricted to the primitive erythrocyte, which circulates only early in chick embryogenesis (day 1-7). The promoter has been sequenced and putative control regions are being analyzed by in vitro footprinting and gel



mobility shift assays. The extracts used for these analyses are derived from erythrocytes obtained at different stages of development spanning the time when the  $\epsilon$  gene is fully active to the adult chicken, when all genes are totally inactive. These regions of differential binding within the  $\epsilon$  promoter are also being analyzed for functional activity by linkage to a reporter gene and subsequent transfection into primary erythrocytes.

HMG 14(a and b) and 17. To establish this system we investigated various parameters related to expression of these genes in erythrocytes during embryogenesis: pulse-labeled protein, steady state mRNA, nuclear run-ons that analyze only newly synthesized transcripts, and chromatin structure of the genes themselves. Our results suggest that the individual genes of the HMG family are differentially expressed in development. HMG 14a, in particular, is restricted to the primitive (early) erythrocyte whereas HMG 14b and 17 are maximally expressed only later, in the definitive erythrocyte, but not in the reticulocyte.

#### Chemical and Structural Investigations of Nucleic Acids and Related Molecules

The DNA dodecamer d-GGTACGGTAC and four derivatives containing GA and IA mispairs in the 6,7 and 5,8 positions were found to have van't Hoff enthalpies from the dependence of  $T_m$  on concentration that appeared to be much too high. Equilibrium centrifugation studies in collaboration with Dr. Ross revealed complex equilibria among duplex, hairpin, and coil. Detailed analysis showed the dependence of the equilibria on concentration, ionic strength, and temperature and provided enthalpies and other thermodynamic parameters of the different transitions. The results suggest that many of the van't Hoff enthalpies reported in the literature for oligonucleotides may be in error.

UV melting of the oligomers in the above series showed a surprising dependence of  $T_m$  on sequence. The 6,7 mispairs had no effect on  $T_m$ , whereas the 5,8 mispairs exhibited large depressions. We attribute these observations to formation of a two-base loop in the basic sequence and the 6,7 mispairs, leaving an identical five-base pair stem for the three oligomers. In the 5,8 mispairs, on the other hand, there is only a four-base pair stem, with resulting lower  $T_m$ .

A two-base hairpin loop, postulated above, has been reported in the literature to be impossible in the ribo series and unstable or unfavorable in DNA. To evaluate the stereochemical feasibility of the two-base loop a theoretical modeling study was carried out in collaboration with Drs. Sasisekharan, Raghunathan, and Jernigan. The calculations showed that a stable two-base loop structure could be formed using standard values of all conformational torsion angles and having no short contacts.

We have found that the drug distamycin binds as well to the homopolymer duplex d2NH<sub>2</sub>Ah·dT as to dA·dT and had previously found that it binds well to the alternating copolymer of d2NH<sub>2</sub>A and dT. These

results indicate that the accepted explanation of failure of the drug to bind GC (that the  $2\text{NH}_2$  group of G interferes sterically with 2  $\text{CH}_2$  residues of the drug) is not correct.

### Thermal Measurements of Biomolecular Systems

We have completed a thermodynamically rigorous computation that elucidates the underlying causes of complex bimodal and polymodal excess heat capacity *vs.* temperature profiles for the thermal denaturation of a macromolecule that may bind an arbitrary number of ligand molecules to either and/or both the native and denatured states between which there may also be a finite difference in heat capacity. These calculations account essentially completely for experimental data we have obtained and demonstrate that complex thermograms can arise from changes in the free ligand concentration and do not necessarily arise from the melting of structural domains within the macromolecule.

We have measured the heat of reaction between the repressor protein cro and six different 21 bp DNA oligonucleotides between 5°C and 37°C. The DNA sequences include two specific operator sequences OR1 and OR3, two completely "non-specific" sequences and two sequences of intermediate affinity. The enthalpy changes are proportional to the affinity for DNA and are endothermic at low temperature becoming exothermic above 25°C. The magnitudes of the enthalpy changes are surprisingly small considering the wide area of contact between protein and DNA.

### Statistical Thermodynamics of Protein and Polynucleotide Systems

The main progress of this project is the development of a general formalism for binding ligands to a one-dimensional lattice to which more than one ligand can bind simultaneously.

### Energy Conversion in Biology

A number of different topics have been studied in the general field of free energy transduction and biophysics of biological systems. The most important areas in which progress has been made are the study of diffusion of lipid-like molecules between membranes of a virus and a lipid bilayer, the application of the first-passage time formalism to the study of kinetics of bond formation in cell-cell adhesion systems, the diagram method in evaluating the exchange fluxes of ion transport systems, the application of muscle contraction formalism to flagellar rotation, and computer simulation study of diffusion-controlled bimolecular recombination on a two-dimensional square lattice.

### Influences of Macromolecular Crowding on Biochemical Systems

*In vitro* studies have demonstrated relatively large excluded volume effects upon a variety of biochemical reactions. Given the high concentrations of macromolecules within living cells, it is anticipated that such excluded volume effects in cells will cause wide-spread changes in rates and equilibria of a host of cellular reactions. We have devised experimental procedures to help estimate the magnitudes of these effects *in vivo*. The goal is the correction of *in vitro* parameters to values

more appropriate to cellular conditions.

We have initially studied the cytoplasmic compartment of E.coli. Our approach is to measure excluded volume parameters in extracts of spheroplasts and cells of E.coli and to correct these parameters for the dilution of macromolecules in the extract relative to the cytoplasm of the cells. This approach has required the development of two new procedures, namely an assay suitable for the estimation of excluded volume parameters in complex mixtures such as cell extracts based upon a two-phase partition system, and also a procedure for correction of extract concentrations back to cytoplasmic conditions.

In an unrelated study which arose from earlier measurements of crowding reactions, we have developed a method for quantitating reactions between specific members of a set of DNA restriction fragments. The method should be generally applicable to reactions involving ligation or site-specific cleavage of specific restriction fragments.

#### Studies of Immunoglobulin Gene Rearrangement

The influence of various chemical effectors on V(D)J recombination in early lymphoid cells has been studied. The test system uses recombinational target sequences on extrachromosomal plasmids which provide a good assay of overall V(D)J recombinase activity in the cells. Several effectors whose common focus is an ultimate increase in the cellular level of cyclic AMP(cAMP) produce large increases in V(D)J recombination (3 to 8-fold). These compounds—caffeine, theophylline, forskolin, and 8-bromo-cAMP—act in different ways but produce similar results. Because the major effect of cAMP in mammalian cells is the stimulation of protein kinase A, effectors of other protein kinase were also studied. Phorbol myristate acetate, a stimulator of protein kinase C, reduces recombination about 4-fold, and a similar effect is seen with the calcium ionophore A23187, a stimulator of both protein kinase C and the calcium/calmodulin dependent protein kinase.

We conclude that V(D)J recombination activity is strongly influenced by chemical signaling pathways.

#### Studies of Functions Involved in Genetic Recombination

The ATP-binding site of DNA gyrase (on the B subunit) has been located by use of an affinity label. The reagent adenylyl-pyridoxal phosphate labels two lysines at positions 103 and 110 in a region with weak homology to some other ATPases.

Another ATP analog, ( $\beta$ ,  $\gamma$ -imido) ATP, which inhibits DNA gyrase by binding reversibly, has been shown to bind very slowly to the enzyme, in contrast to ATP whose binding is rapid.

#### Studies on the Mechanism of Genetic Recombination

The major objective of this project is to uncover the enzymatic steps involved in various genetic rearrangement reactions and to study

the mechanism of action of the enzymes involved. The mechanisms of the transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate a branched DNA intermediate. Efficient formation of this intermediate requires Mu A, Mu B and *E. coli* HU proteins along with ATP and  $Mg^{++}$ . The Mu A protein binds to the Mu end DNA sequences on the donor DNA by one domain and to the internal Mu operator DNA site by another domain to form a special protein-DNA complex necessary to the initiation of Mu DNA strand transfer reaction. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence to yield cleaved donor DNA with tightly associated Mu A proteins. This protein-DNA complex captures a second DNA molecule efficiently provided it is bound by Mu B protein. A staggered cut is introduced into the target DNA and each 5' end is joined to each of the 3' ends of the Mu end sequences by a concerted DNA cutting and joining reaction. Evidence has been obtained that this step of the reaction takes place by one step transesterification mechanism.

The Mu B protein, an ATPase, selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The Mu B protein dissociates selectively from the DNA molecule to which Mu A protein is bound in a process that depends on hydrolysis of ATP. Kinetic aspects of this energy transduction system are studied.

#### Studies of the Mechanism of Retroviral DNA Integration

Integration of a DNA copy of a retroviral genome into a chromosome of an infected cell is an essential step for normal viral replication. Our objectives are to analyze the detailed molecular mechanism of this DNA integration reaction and to develop simple cell-free assay systems that may be used to screen for drugs that inhibit this step of the viral replication cycle.

Retroviral DNA integration involves two central steps, cleavage of two nucleotides from the 3' ends of the viral DNA and subsequent joining of these processed 3' ends to the 5' ends of a staggered cut made in the target DNA. We have determined with both the Moloney murine leukemia virus (MoMLV) and HIV systems that a single viral protein accomplishes these reactions. This protein is the IN protein which is encoded at the 3' end of the viral *pol* gene. Our current research therefore focusses on the biochemical activities of the MoMLV and HIV IN proteins. The MoMLV IN protein has been expressed in insect cells and partially purified. MoMLV IN protein has a site-specific nuclease activity that cleaves two nucleotides from the sequences present at the ends of MoMLV DNA. This reaction generates the recessed 3' ends that the precursors for integration. MoMLV IN protein also accomplishes the subsequent step of integrating these ends into a target DNA. We have expressed the HIV IN protein in insect cells and shown that it has the same biochemical activities as the MoMLV IN protein. To provide a more abundant source of HIV IN protein for physical studies we have also expressed this protein in *E. coli* and purified it in active form.

We can now efficiently carry out the two central steps of retroviral DNA integration with cloned MoMLV or HIV IN proteins and oligonucleotide DNA substrates. This assay is being developed to provide a simple and economical means to screen for drugs that inhibit HIV DNA integration.

#### Developmental Regulation of Differential Gene Expression

Our work is focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogenesis. Progress has been achieved in three key areas. First, we have defined the protein-nucleic acid interactions within a complete transcription complex assembled onto a 5S RNA gene. For the first time *in vitro* we have established conditions under which every gene is actively transcribed. Secondly, we have extended our understanding of the role of chromatin structure in preventing transcription factors from associating with genes. We have defined two distinct stages in chromatin assembly on replicating DNA: the addition of histones H3/H4 precedes the deposition of histones H2A/H2B. In both *Xenopus* egg extracts and in a biochemically purified system we have examined the effects of this staged chromatin assembly on transcription. We find that a histone H3/H4/DNA complex is transcriptionally active. Therefore we can account for the transcription of newly replicated DNA and the gradual repression of genes as H2A/H2B are sequestered. Replication and transcription are associated with substantial changes in DNA topology, we find both positive and negative supercoiling to be without effect on 5S RNA gene transcription. We have defined the structure of DNA in nucleosomes at high resolution and mapped nucleosome positions on 5S DNA *in vivo* at low resolution. Finally, a new family of class II gene transcription factors have been cloned, their function analyzed and their developmental regulation studied. These Y-box transcription factors have properties consistent with their having a major role in regulating germ-cell specific transcription.

#### Structural Molecular Biology

Alternative non-B DNA structures may play a role in gene function by modulating interactions with regulatory proteins. We are using two related physical techniques to analyze possible conformations of DNA and DNA-protein complexes.

**Birefringence.** We have analyzed the persistence length of a 250bp fragment of DNA containing the 5S RNA gene of *Xenopus borealis*. In the presence of Zinc (+2) or spermidine this fragment has an altered structure not assumed by a control piece of DNA. This altered structure is a bend centered at +50 to +60, which is the 5' end of the internal control region. We are currently analyzing this 5S RNA gene complexed to TFIID, a protein which binds in the middle of the gene and is essential for transcription.

**Photochemical Electric Dichroism.** We have developed a new technique for analyzing DNA structure and DNA-protein interaction. It involves combination of photochemical techniques (generation of UV pyrimidine dimer crosslinks by laser illumination) with electric

dichroism (orientation of DNA in a high electric field). We have analyzed the 5S RNA gene of sea urchins and have found an alternate DNA structure associated with oligopurine tracts within the TFIID-binding domain of this gene.

#### Replication, Recombination and Repair of Microbial DNA

Studies on DNA replication of plasmid ColE1 have been continued. Transcripts (RNA II) that start 555 nucleotides upstream of the replication origin by RNA polymerase form hybrids with the template DNA. The hybridized transcript is cleaved by ribonuclease H at the origin and used as the primer for leading strand synthesis of DNA polymerase I. Primer formation is regulated by a plasmid-specified small RNA (RNA I), which is transcribed from the DNA coding 5' end region of RNA II, but in the direction opposite to that of RNA II synthesis. This antisense RNA binds to RNA II and prevents RNA II to form the secondary structure necessary for primer formation. The binding begins with interaction between loops of RNAs. Isolated complementary single stem-loops of these RNAs also form complex, in which all the bases in the loops form base-pairing. The stability is determined by both the base-pairing between loops and the base-stacking with neighboring bases. Primer formation is also regulated by a 63-amino acid protein specified by the plasmid. The protein called Rom binds to a very unstable initial complex made between RNA I and RNA II and thus enhances the inhibitory action of RNA I. A single dimer molecule of Rom binds to the complex formed by complementary single stem-loops by recognizing the structure rather than its exact nucleotide sequence. The binding of Rom stabilizes the complex by decreasing the rate of dissociation of the complex without affecting the rate of association of the RNA components. Inhibition of primer formation by RNA I in the presence or absence of the protein determines the copy number of a plasmid in a cell and the incompatibility between related plasmids.

#### Mammalian DNA Replication, Regulation and Amplification

We have concluded an analysis of sequences thought to be enriched for mammalian replication origins and report them to be enriched for (i) a particular transcription regulatory element, (ii) AT-rich sequences (iii) matrix attachment regions and (iv) the ARS consensus sequence for S. cerevisiae but not S. pombe.

#### Channeling in the Biosynthesis of Histidine

A series of 8 enzymatic reactions are responsible for the conversion of ATP and PRPP to histidine. Curiously, two of the reactions which are non-sequential, are carried out by the same protein. Is this mere happenstance or a suggestion that all of the proteins in this series interact to form a complex? Although sucrose gradient centrifugate was developed precisely to ask this question, the negative result may merely reflect the enormous dilution involved in these assays. New techniques of molecular crowding and cooperative enzyme kinetics will be employed to readdress this question.

### Nonheritable Antibiotic Resistance

The mechanisms whereby salicylates and some related compounds alter the antibiotic resistances of *E. coli* have been further elucidated. In collaboration with S. Levy, we have found that salicylate-treated cells are similar to certain marA mutants. Salicylate-treated cells are multiply antibiotic resistant, have outer membranes with decreased permeability to cephalosporins, have reduced levels of the outer membrane protein OmpF, and have increased levels of micF mRNA which is antisense to and which down-regulates ompF translation. marA mutants overexpress three adjacent ORFs (in the absence of salicylate) and this, somehow, leads to the same phenotype found for salicylate-treated cells. We have now shown that salicylate stimulates the expression of the marA operon, whether normal or mutant. Thus, the similarities between salicylate-treated cells and marA mutants is likely to be due to this common affect on marA expression.

An effect of salicylate (and the non-acidic, salicyl alcohol) that is not observed with marA mutants is that they increase *E. coli*'s sensitivity to various positively charged antimicrobials: aminoglycosides, bleomycin and  $\text{Cd}^{++}$ . Two uptake systems for  $\text{Cd}^{++}$  were observed in untreated cells. Treatment with salicylate or salicyl alcohol increased the  $V_{\text{max}}$  but did not change the  $K_m$  of these systems. The clear correlation between increased sensitivity and increased uptake of  $\text{Cd}^{++}$  suggests that the mechanism of salicylate action in this case is to increase the uptake of certain cations.

### Regulation of a Gene Expressed in Undifferentiated Teratocarcinoma Cells

The crypto gene was found by serendipity in a screening of a cDNA library from human teratocarcinoma cells. The gene is expressed only in undifferentiated teratocarcinoma cells and shuts off on exposure of the cells to retinoic acid which induces differentiation. The gene is expressed in mouse teratocarcinoma cells and behaves similarly. The sequence of the gene looks remarkably like the growth factor EGF, but is clearly distinct from that factor in its expression. When placed under the control of an RSV promoter, crypto gene expression results in the transformation of certain but not all cell types. Crypto gene expression has been found only in certain basal cell carcinomas. It was also found in an expression library made from early blastula rat embryos. On the other hand 11 day embryos showed no detectable levels of expression. A baculovirus expression vector has been made.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Zol DK 33000-24 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Functions Involved in Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Martin Gellert Chief, Section on Metabolic Enzymes LMB/NIDDK

Others: James K. Tamura Staff Fellow LMB/NIDDK  
Andrew Bates Visiting Fellow LMB/NIDDK  
Mary H. O'Dea Research Chemist LMB/NIDDK

COOPERATING UNITS (if any)

Dr. G. Zaccai, Institut Max Von Laue-Paul Langevin, Grenoble, France  
Dr. A. Maxwell, University of Leicester, Leicester, U.K.  
Ms. S. Krueger, University of Maryland, College Park, MD

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.7

PROFESSIONAL:

2.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The ATP-binding site of DNA gyrase (on the B subunit) has been located by use of an affinity label. The reagent adenylyl-pyridoxal phosphate labels two lysines at positions 103 and 110 in a region with weak homology to some other ATPases.

Another ATP analog, ( $\beta$ ,  $\gamma$ -imido) ATP, which inhibits DNA gyrase by binding reversibly, has been shown to bind very slowly to the enzyme, in contrast to ATP whose binding is rapid.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33001-6 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Immunoglobulin Gene Rearrangement

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Kiyoshi Mizuuchi	Chief, Section on Genetic Mechanisms	LMB/NIDDK

Others: David Brown	Clinical Associate	LMB/NIDDK
Joanne Hesse	Research Chemist	LMB/NIDDK
Fraser McBlane	Visiting Fellow	LMB/NIDDK
Joseph Menetski	Guest Researcher	LMB/NIDDK
Moshe Sadofsky	Guest Worker	LMB/NIDDK

## COOPERATING UNITS (if any)

Dr. Susanna Lewis, California Institute of Technology  
Dr. David Roth, LP/NCI

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Metabolic Enzymes

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

5.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The influence of various chemical effectors on V(D)J recombination in early lymphoid cells has been studied. The test system uses recombinational target sequences on extrachromosomal plasmids which provide a good assay of overall V(D)J recombinase activity in the cells. Several effectors whose common focus is an ultimate increase in the cellular level of cyclic AMP(cAMP) produce large increases in V(D)J recombination (3 to 8-fold). These compounds--caffeine, theophylline, forskolin, and 8-bromo-cAMP--act in different ways but produce similar results. Because the major effect of cAMP in mammalian cells is the stimulation of protein kinase A, effectors of other protein kinase were also studied. Phorbol myristate acetate, a stimulator of protein kinase C, reduces recombination about 4-fold, and a similar effect is seen with the calcium ionophore A23187, a stimulator of both protein kinase C and the calcium/calmodulin dependent protein kinase.

We conclude that V(D)J recombination activity is strongly influenced by chemical signaling pathways.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33002-04 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of DNA Supercoiling on the Topological Properties of Nucleosomes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Martin Gellert Chief, Section on Metabolic Enzymes LMB/NIDDK  
Gary Felsenfeld Chief, Section on Physical Chemistry LMB/NIDDK

Others: David Clark Visiting Fellow LMB/NIDDK  
Mary H. O'Dea Research Chemist LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Metabolic Enzymes

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0

## PROFESSIONAL:

0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project was terminated during FY 1990.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33006-12 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mechanism of Genetic Recombination

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kiyoshi Mizuuchi Chief, Section on Genetic Mechanisms LMB/NIDDK

Others: K. Adzuma Visiting Associate LMB/NIDDK  
M. Mizuuchi Visiting Associate LMB?NIDDK  
T. Baker Special Volunteer

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Genetic Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major objective of this project is to uncover the enzymatic steps involved in various genetic rearrangement reactions and to study the mechanism of action of the enzymes involved. The mechanisms of the transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate a branched DNA intermediate. Efficient formation of this intermediate requires Mu A, Mu B and E. coli HU proteins along with ATP and  $Mg^{++}$ . The Mu A protein binds to the Mu end DNA sequences on the donor DNA by one domain and to the internal Mu operator DNA site by another domain to form a special protein-DNA complex necessary to the initiation of Mu DNA strand transfer reaction. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence to yield cleaved donor DNA with tightly associated Mu A proteins. This protein-DNA complex captures a second DNA molecule efficiently provided it is bound by Mu B protein. A staggered cut is introduced into the target DNA and each 5' end is joined to each of the 3' ends of the Mu end sequences by a concerted DNA cutting and joining reaction. Evidence has been obtained that this step of the reaction takes place by one step transesterification mechanism.

The Mu B protein, an ATPase, selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The Mu B protein dissociates selectively from the DNA molecule to which Mu A protein is bound in a process that depends on hydrolysis of ATP. Kinetic aspects of this energy transduction system are studied.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34001-25 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Gary Felsenfeld, Chief, Section on Physical Chemistry LMB/NIDDK

OTHERS:

David Clark, Visiting Associate	LMB/NIDDK	Catherine Lewis, Sen. Staff Fellow	LMB/NIDDK
Todd Evans, Staff Fellow	LMB/NIDDK	Mark Minie, Staff Fellow	LMB/NIDDK
Gretchen Gibney, Special Volunteer	LMB/NIDDK	Joanne Nickol, Research Chemist	LMB/NIDDK
Hannah Gould, Expert	LMB/NIDDK	Marc Reitman, Sen. Staff Fellow	LMB/NIDDK
Joseph Knezetic, Staff Fellow	LMB/NIDDK	Cecelia Trainor, IRTA Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Department of Biophysics, King's College (Robert Hannon)  
Nat'l Inst. of Child Health and Human Develop., NIH (Heiner Westphal)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

8.5

PROFESSIONAL:

8.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have continued to study the role played by the general erythroid-specific factor which we have named Eryf1. The cDNA for Eryf1 has been cloned, and we have studied the ability of the protein to serve as a transactivating factor in a variety of cells. The fact that very high levels of transcriptional stimulation are observed in certain cell types provides important information about the mechanism of activation by Eryf1. We have also cloned the cDNA for the human Eryf1. Comparison of the structure and activity of chicken and human proteins provides information both about their evolution and function. To learn more about factors that may act directly on chromatin structure, we have characterized in greater detail the binding of the factor BGPI, which binds to a string of 16 G residues in the adult  $\beta$ -globin hypersensitive domain. We have also surveyed chromatin of the entire  $\beta$ -globin domain for nuclease hypersensitive sites. This revealed four candidates for roles as dominant control regions (DCRs). Subsequent experiments in transgenic mice reveal that one of these, the  $\beta$ -globin enhancer already characterized in our laboratory, has the properties of a DCR. This provides an important potential connection between classical regulatory elements and chromatin structure.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 34002-26 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzyme Structure

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

**PI:** David R. Davies, Chief, Section on Molecular Structure LMB/NIDDK  
**Others:** C. Craig Hyde, Senior Staff Fellow LMB/NIDDK  
Kevin Parris, IRTA LMB/NIDDK  
John R.H. Gilstad, Guest Researcher LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Molecular Structure

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.25

## PROFESSIONAL:

2.25

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

## Summary:

- 1) The crystal structure of the bifunctional enzyme complex, tryptophan synthase, has been refined at 2.4Å resolution.
- 2) Two inhibitor complexes of the Rhizopuspepsin have been analyzed by X-ray diffraction. Both complexes have been refined and the structures analyzed in terms of the probable mechanism of action.
- 3) Endonexin II, a calcium- and phospholipid-binding protein isolated from human placenta, has been successfully crystallized. X-ray diffraction data have been collected from native and heavy-atom treated crystals, and their space group has been determined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34003-22 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Three-Dimensional Structure of Proteins of the Immune System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	David R. Davies, Chief, Section on Molecular Structure	LMB/NIDDK
Others:	Eduardo A. Padlan, Visiting Scientist	LMB/NIDDK
	Gerson H. Cohen, Research Chemist	LMB/NIDDK
	Enid W. Silverton, Research Chemist	LMB/NIDDK
	Boaz Shaanan, Visiting Scientist	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Structure

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4

PROFESSIONAL:

4

OTHER:

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Summary:

- 1) The structure of the McPC603 Fab has been completely refined.
- 2) The structure of the McPC603 Fab-phosphocholine complex has been completely refined.
- 3) The crystal structure of a lactose specific lectin has been analyzed and almost completely refined.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 35000-26 LMB

PERIOD COVERED  
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H. Todd Miles, Chief, Section on Organic Chemistry LMB/NIDDK

Others: F. B. Howard Research Chemist LMB/NIDDK  
J. Frazier Research Chemist LMB/NIDDK

COOPERATING UNITS (if any) Girjesh Govil TIFR, Bombay, India / Philip ROSS LMB/NIDDK  
V. Sasisekharan Fogarty Scholar; Indian Institute of Science, Bangalore, India  
N. Patibhiraman Naval Research Laboratory, Washington, D. C.  
G. Raghunathan NCI / R. L. Jernigan, NCI  
C-Q Chen Biotechnology Center, Shanghai

LAB/BRANCH Laboratory of Molecular Biology

SECTION Section on Organic Chemistry

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 4.5 PROFESSIONAL: 4.5 OTHER: 0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The DNA dodecamer d-GGTACGCGTACC and four derivatives containing GA and IA mispairs in the 6,7 and 5,8 positions were found to have van't Hoff enthalpies from the dependence of  $T_m$  on concentration that appeared to be much too high. Equilibrium centrifugation studies in collaboration with Dr. Ross revealed complex equilibria among duplex, hairpin, and coil. Detailed analysis showed the dependence of the equilibria on concentration, ionic strength, and temperature and provided enthalpies and other thermodynamic parameters of the different transitions. The results suggest that many of the van't Hoff enthalpies reported in the literature for oligonucleotides may be in error.

UV melting of the oligomers in the above series showed a surprising dependence of  $T_m$  on sequence. The 6,7 mispairs had no effect on  $T_m$ , whereas the 5,8 exhibited large depressions. We attribute these observations to formation of two-base loop in the basic sequence and the 6,7 mispairs, leaving an identical five-base pair stem for the three oligomers. In the 5,8 mispairs, on the other hand, there is only a four-base pair stem, with resulting lower  $T_m$ .

A two-base hairpin loop, postulated above, has been reported in the literature to be impossible in the ribo series and unstable or unfavorable in DNA. To evaluate the stereochemical feasibility of a two-base loop a theoretical modeling study was carried out in collaboration with Drs. Sasisekharan, Raghunathan, and Jernigan. The calculations showed that a stable two-base loop structure could be formed using standard values conformational torsion angles and having no short contacts.

We have found that the drug distamycin binds as well to the homopolymer duplex d2NH<sub>2</sub>A·dT as to dA·dT and had previously found that both drugs bind well to the alternating copolymer of d2NH<sub>2</sub>A and dT. These results indicate that the accepted explanation of failure to bind GC (that the 2NH<sub>2</sub> group of G interferes sterically with 2 CH<sub>2</sub> residues of the drug) is not correct.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 35050-19 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Replication, Recombination and Repair of Microbial DNA

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Tomizawa Chief, Section on Molecular Genetics LMB/NIDDK

Others: Y. Eguchi Visiting Fellow

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Molecular Genetics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on DNA replication of plasmid ColE1 have been continued. Transcripts (RNA II) that start 555 nucleotides upstream of the replication origin by RNA polymerase form hybrids with the template DNA. The hybridized transcript is cleaved by ribonuclease H at the origin and used as the primer for leading strand synthesis of DNA polymerase I. Primer formation is regulated by a plasmid-specified small RNA (RNA I), which is transcribed from the DNA coding 5' end region of RNA II, but in the direction opposite to that of RNA II synthesis. This antisense RNA binds to RNA II and prevents RNA II to form the secondary structure necessary for primer formation. The binding begins with interaction between loops of RNAs. Isolated complementary single stem-loops of these RNAs also form complex, in which all the bases in the loops form base-pairing. The stability is determined by both the base-pairing between loops and the base-stacking with neighboring bases. Primer formation is also regulated by a 63-amino acid protein specified by the plasmid. The protein called Rom binds to a very unstable initial complex made between RNA I and RNA II and thus enhances the inhibitory action of RNA I. A single dimer molecule of Rom binds to the complex formed by complementary single stem-loops by recognizing the structure rather than its exact nucleotide sequence. The binding of Rom stabilizes the complex by decreasing the rate of dissociation of the complex without affecting the rate of association of the RNA components. Inhibition of primer formation by RNA I in the presence or absence of the protein determines the copy number of a plasmid in a cell and the incompatibility between related plasmids.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36003-6 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nonheritable Antibiotic Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.L. Rosner Research Biologist LMB/NIDDK  
Others: J.D. Foulds Research Chemist LSB/NIDDK  
M. Zasloff Pediatric Physician Children's Hospital  
Philadelphia, PA  
M. Aumercier Visiting Fellow LMB/NIDDK

COOPERATING UNITS (if any)

Foreign: Maria Persico, Senior Scientist, Int. Lab. of Gen. & Biophysics, Naples, Italy

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.25

PROFESSIONAL:

2.25

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanisms whereby salicylates and some related compounds alter the antibiotic resistances of E. coli have been further elucidated. In collaboration with S. Levy, we have found that salicylate-treated cells are similar to certain marA mutants. Salicylate-treated cells are multiply antibiotic resistant, have outer membranes with decreased permeability to cephalosporins, have reduced levels of the outer membrane protein OmpF, and have increased levels of micF mRNA which is antisense to and which down-regulates ompF translation. marA mutants overexpress three adjacent ORFs (in the absence of salicylate) and this, somehow, leads to the same phenotype found for salicylate-treated cells. We have now shown that salicylate stimulates the expression of the marA operon, whether normal or mutant. Thus, the similarities between salicylate-treated cells and marA mutants is likely to be due to this common affect on marA expression.

An effect of salicylate (and the non-acidic, salicyl alcohol) that is not observed with marA mutants is that they increase E. coli's sensitivity to various positively charged antimicrobials; aminoglycosides, bleomycin and  $Cd^{++}$ . Two uptake systems for  $Cd^{++}$  were observed in untreated cells. Treatment with salicylate or salicyl alcohol increased the  $V_{max}$  but did not change the  $K_m$  of these systems. The clear correlation between increased sensitivity and increased uptake of  $Cd^{++}$  suggests that the mechanism of salicylate action in this case is to increase the uptake of certain cations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36051-22 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mammalian DNA Replication, Regulation and Amplification

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert G. Martin, Chief, Sec. on Microbial Genetics LMB/NIDDK

Others: B. S. Rao Visiting Fellow LMB/NIDDK

M. Reitman Senior Staff Fellow LMB/NIDDK

## COOPERATING UNITS (if any)

Foreign: M. Zannis-Hadjopoulos, McGill Cancer Center, Montreal, Canada  
G. Kaufmann, Tel Aviv University, Tel Aviv, Israel  
H. Manor, Technicon University, Haifa, Israel

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Microbial Genetics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have concluded an analysis of sequences thought to be enriched for mammalian replication origins and report them to be enriched for (i) a particular transcription regulatory element, (ii) AT-rich sequences (iii) matrix attachment regions and (iv) the ARS consensus sequence for S. cerevisiae but not S. pombe.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 36101-16 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Conversion in Biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Yi-der Chen Research Chemist LMB/NIDDK

Others: Robert J. Rubin Special Volunteer LMB/NIDDK

## COOPERATING UNITS (if any)

Shahid Khan, Albert Einstein College of Medicine, NY, NY 10461  
Aydin Tozeren, Catholic University, Washington, D. C. 20064

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.80

## PROFESSIONAL:

.80

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

A number of different topics have been studied in the general field of free energy transduction and biophysics of biological systems. The most important areas in which progress has been made are the study of diffusion of lipid-like molecules between membranes of a virus and a lipid bilayer, the application of the first-passage time formalism to the study of kinetics of bond formation in cell-cell adhesion systems, the diagram method in evaluating the exchange fluxes of ion transport systems, the application of muscle contraction formalism to flagellar rotation, and computer simulation study of diffusion-controlled bimolecular recombination on a two-dimensional square lattice.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36102-19 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Thermodynamics of Protein and Polynucleotide Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Yi-der Chen Research Chemist LMB/NIDDK

## COOPERATING UNITS (if any)

J. Chalovich, University East Carolina, North Carolina

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.20

## PROFESSIONAL:

.20

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The main progress of this project is the development of a general formalism for binding ligands to a one-dimensional lattice to which more than one ligand can bind simultaneously.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36104-09 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermal Measurements of Biomolecular Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: P.D. Ross Research Chemist LMB/NIDDK

OTHERS:

A.C. Steven	Visiting Scientist	LPB/NIAMS	M.S. Lewis	Research Chemist	BEIP/NCRR
A. Shrake	Research Chemist	DBBP/CPB	Y. Takeda	Research Chemist	FCRF/NCI
F.B. Howard	Research Chemist	LMB/NIDDK	C.P. Mudd	Research Chemist	BEI/DRS
H.T. Miles	Research Chemist	LMB/NIDDK			

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemsitry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. We have carried out an equilibrium ultracentrifugation study of five DNA dodecamers four of which contain purine-purine mispairs. At temperatures below the main order-disorder transition, solutions of these DNA molecules are equilibrium mixtures of organized monomeric hairpin and double helical molecules. The finding of hairpin molecules has: a) led to the inference based upon optical melting data that two base pair loops are possible in DNA b) led to a satisfactory and self-consistent thermodynamic analysis of the melting transition which by inclusion of the helix-hairpin equilibrium results in reasonable values of the associated thermodynamic parameters as opposed to unreasonable values obtained by assuming an oversimplified two-state model for the transition.
2. We have completed a thermodynamically rigorous computation that elucidates the underlying causes of complex bimodal and polymodal excess heat capacity vs. temperature profiles for the thermal denaturation of a macromolecule that may bind an arbitrary number of ligand molecules to either and/or both the native and denatured states between which there may also be a finite difference in heat capacity. These calculations account essentially completely for experimental data we have obtained and demonstrate that complex thermograms can arise from changes in the free ligand concentration and do not necessarily arise from the melting of structural domains within the macromolecule.
3. We have measured the heat of reaction between the repressor protein cro and six different 21 bp DNA oligonucleotides between 5°C and 37°C. The DNA sequences include two specific operator sequences OR1 and OR3, two completely "non-specific" sequences and two sequences of intermediate affinity. The enthalpy changes are proportional to the affinity for DNA and are endothermic at low temperature becoming exothermic above 25°C. The magnitudes of the enthalpy changes are surprisingly small considering the wide area of contact between protein and DNA.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36105-08 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Influences of Macromolecular Crowding on Biochemical Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.B. Zimmerman

Research Chemist

LMB/NIDDK

## OTHERS:

S.O. Trach

Research Chemist

LMB/NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In vitro studies have demonstrated relatively large excluded volume effects upon a variety of biochemical reactions. Given the high concentrations of macromolecules within living cells, it is anticipated that such excluded volume effects in cells will cause wide-spread changes in rates and equilibria of a host of cellular reactions. We have devised experimental procedures to help estimate the magnitudes of these effects in vivo. The goal is the correction of in vitro parameters to values more appropriate to cellular conditions.

We have initially studied the cytoplasmic compartment of E.coli. Our approach is to measure excluded volume parameters in extracts of spheroplasts and cells of E.coli and to correct these parameters for the dilution of macromolecules in the extract relative to the cytoplasm of the cells. This approach has required the development of two new procedures, namely an assay suitable for the estimation of excluded volume parameters in complex mixtures such as cell extracts based upon a two-phase partition system, and also a procedure for correction of extract concentrations back to cytoplasmic conditions.

In an unrelated study which arose from earlier measurements of crowding reactions, we have developed a method for quantitating reactions between specific members of a set of DNA restriction fragments. The method should be generally applicable to reactions involving ligation or site-specific cleavage of specific restriction fragments.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36106-03 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Developmental Regulation of Differential Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alan Wolffe, Visiting Scientist LMB/NIDDK

## OTHERS:

Constantin Chipev, Visiting Associate	LMB/NIDDK
Mary Familari, Visiting Fellow	LMB/NIDDK
Jeffrey Hayes, Special Volunteer	LMB/NIDDK (NRC Fellow)
Sherrie Tafuri, IRTA Fellow	LMB/NIDDK

## COOPERATING UNITS (if any)

Biol. Div., Oak Ridge Nat'l Lab. (D.E. Olins); Dept. Chem., Johns Hopkins Univ. (T. Tullius); Lab. of Mol. Carcinogenesis NCI (M. Bustin, M. Crippa); Div. of Biotech. CSIRO, Sydney, Australia (H.R. Drew); Inst. Jacques Monod, Paris, France (M. Mechali, G. Almouzni); Inst. Biologie Animale, Lausanne, Switzerland (W. Wahli, C. Schild); Lab. of Cell. and Devel. Biol., NIDDK (R. Morse); Dept. Biol., Washington Univ. (S. Elgin)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.8

## PROFESSIONAL:

3.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our work is focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogenesis. Progress has been achieved in three key areas. First, we have defined the protein-nucleic acid interactions within a complete transcription complex assembled onto a 5S RNA gene. For the first time in vitro we have established conditions under which every gene is actively transcribed. Secondly, we have extended our understanding of the role of chromatin structure in preventing transcription factors from associating with genes. We have defined two distinct stages in chromatin assembly on replicating DNA: the addition of histones H3/H4 precedes the deposition of histones H2A/H2B. In both Xenopus egg extracts and in a biochemically purified system we have examined the effects of this staged chromatin assembly on transcription. We find that a histone H3/H4/DNA complex is transcriptionally active. Therefore we can account for the transcription of newly replicated DNA and the gradual repression of genes as H2A/H2B are sequestered. Replication and transcription are associated with substantial changes in DNA topology, we find both positive and negative supercoiling to be without effect on 5S RNA gene transcription. We have defined the structure of DNA in nucleosomes at high resolution and mapped nucleosome positions on 5S DNA in vivo at low resolution. Finally, a new family of class II gene transcription factors have been cloned, their function analyzed and their developmental regulation studied. These Y-box transcription factors have properties consistent with their having a major role in regulating germ-cell specific transcription.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 36108-03 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Mechanism of Retroviral DNA Integration

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Robert Craigie	Visiting Scientist	LMB/NIDDK
	Kiyoshi Mizuuchi	Chief, Section on Genetic Mechanisms	LMB/NIDDK
	Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Others:	Frederic Bushman	Special Volunteer	LMB/NIDDK
	Alan Engelman	IRTA	LMB/NIDDK
	Myung-Soo Lee	Special Volunteer	LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Genetic Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

3

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Integration of a DNA copy of a retroviral genome into a chromosome of a infected cell is an essential step for normal viral replication. Our objectives are to analyze the detailed molecular mechanism of this DNA integration reaction and to develop simple cell-free assay systems that may be used to screen for drugs that inhibit this step of the viral replication cycle.

Retroviral DNA integration involves two central steps, cleavage of two nucleotides from the 3' ends of the viral DNA and subsequent joining of these processed 3' ends to the 5' ends of a staggered cut made in the target DNA. We have determined with both the Moloney murine leukemia virus (MoMLV) and HIV systems that a single viral protein accomplishes these reactions. This protein is the IN protein which is encoded at the 3' end of the viral *pol* gene. Our current research therefor focusses on the biochemical activities of the MoMLV and HIV IN proteins. The MoMLV IN protein has been expressed in insect cells and partially purified. MoMLV IN protein has a site-specific nuclease activity that cleaves two nucleotides from the sequences present at the ends of MoMLV DNA. This reaction generates the recessed 3' ends that the precursors for integration. MoMLV IN protein also accomplishes the subsequent step of integrating these ends into a target DNA. We have expressed the HIV IN protein in insect cells and shown that it has the same biochemical activities as the MoMLV IN protein. To provide a more abundant source of HIV IN protein for physical studies we have also expressed this protein in *E. coli* and purified it in active form.

We can now efficiently carry out the two central steps of retroviral DNA integration with cloned MoMLV or HIV IN proteins and oligonucleotide DNA substrates. This assay is being developed to provide a simple and economical means to screen for drugs that inhibit HIV DNA integration.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36109-3 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

AIDS Related Proteins: Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David R. Davies, Chief, Section on Molecular Structure  
Others: Enid W. Silverton, Research Chemist  
Christina J. Brown, Visiting Fellow  
Arthur B. Shaw, Expert  
Jim Steinberg, Summer Student

LMB/NIDDK  
LMB/NIDDK  
LMB/NIDDK  
LMB/NIDDK  
LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.74

PROFESSIONAL:

2.5

OTHER:

.24

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews

☐ (b) Human tissues

☒ (c) Neither

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Summary:

- 1) A HIV RNase H construct has been crystallized. X-ray diffraction studies on the crystals show very weak diffraction to low resolution only.
- 2) Small crystals of human soluble CD4 complexed with the antibody OKT4a Fab have been obtained.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36110-02 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Gene Expression during Chicken Erythrocyte Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joanne Nickol, Research Chemist - LMB/NIDDK

COOPERATING UNITS (if any)

Salk Institute (B. Emerson)  
Lab. of Molecular Carcinogenesis, NICK, NIH (M. Crippa, M. Bustin)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

.50

PROFESSIONAL:

.50

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Developmental regulation of expression of two major gene families was studied: globin genes and HMG 14(a and b) and 17 genes. The former represents a differentiated function restricted to erythrocytes whereas the latter genes are constitutively expressed as an essential component of cellular chromatin.

A. Globin genes. Expression of the  $\epsilon$  globin gene is restricted to the primitive erythrocyte, which circulates only early in chick embryogenesis (day 1-7). The promoter has been sequenced and putative control regions are being analyzed by *in vitro* footprinting and gel mobility shift assays. The extracts used for these analyses are derived from erythrocytes obtained at different stages of development spanning the time when the  $\epsilon$  gene is fully active to the adult chicken, when all genes are totally inactive. These regions of differential binding within the  $\epsilon$  promoter are also being analyzed for functional activity by linkage to a reporter gene and subsequent transfection into primary erythrocytes.

B. HMG 14(a and b) and 17. To establish this system we investigated various parameters related to expression of these genes in erythrocytes during embryogenesis: pulse-labeled protein, steady state mRNA, nuclear run-ons that analyze only newly synthesized transcripts, and chromatin structure of the genes themselves. Our results suggest that the individual genes of the HMG family are differentially expressed in development. HMG 14a, in particular, is restricted to the primitive (early) erythrocyte whereas HMG 14b and 17 are maximally expressed only later, in the definitive erythrocyte, but not in the reticulocyte.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36111-02 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Molecular Biology

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joanne Nickol, Research Chemist LMB/NIDDK

## COOPERATING UNITS (if any)

Laboratory of Biochemical Metabolism, NIDDK, NIH (D. Rau);  
 University of Calgary Medical School, Calgary, Alberta, Canada (D. Bazett Jones,  
 M. Brown).

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.25

## PROFESSIONAL:

.25

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Alternative non-B DNA structures may play a role in gene function by modulating interactions with regulatory proteins. We are using two related physical techniques to analyze possible conformations of DNA and DNA-protein complexes.

A. Birefringence. We have analyzed the persistence length of a 250bp fragment of DNA containing the 5S RNA gene of *Xenopus borealis*. In the presence of Zinc (+2) or spermidine this fragment has an altered structure not assumed by a control piece of DNA. This altered structure is a bend centered at +50 to +60, which is the 5' end of the internal control region. We are currently analyzing this 5S RNA gene complexed to TFI<sub>II</sub>A, a protein which binds in the middle of the gene and is essential for transcription.

B. Photochemical Electric Dichroism. We have developed a new technique for analyzing DNA structure and DNA-protein interaction. It involves combination of photochemical techniques (generation of UV pyrimidine dimer crosslinks by laser illumination) with electric dichroism (orientation of DNA in a high electric field). We have analyzed the 5S RNA gene of sea urchins and have found an alternate DNA structure associated with oligopurine tracts within the TFI<sub>II</sub>A-binding domain of this gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36112-2 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of a Gene Expressed in Undifferentiated Teratocarcinoma Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Persico Visiting Scientist IMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Microbial Genetics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.3

## PROFESSIONAL:

.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Crypto Gene was found by serendipity in a screening of a cDNA library from human teratocarcinoma cells. The gene is expressed only in undifferentiated teratocarcinoma cells and shuts off on exposure of the cells to retinoic acid which induces differentiation. The gene is expressed in mouse teratocarcinoma cells and behaves similarly. The sequence of the gene looks remarkably like the growth factor EGF, but is clearly distinct from that factor in its expression. When placed under the control of an RSV promoter, crypto gene expression results in the transformation of certain but not all cell types. Crypto gene expression has been found only in certain basal cell carcinomas. It was also found in an expression library made from early blastula rat embryos. On the other hand 11 day embryos showed no detectable levels of expression. A baculovirus expression vector has been made.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36113-1 LMB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Channeling in the Biosynthesis of Histidine

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert G. Martin, Chief Sec. on Microbial Genetics, LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Microbial Genetics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.50

## PROFESSIONAL:

.50

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A series of 8 enzymatic reactions are responsible for the conversion of ATP and PRPP to histidine. Curiously, two of the reactions, which are non-sequential, are carried out by the same protein. Is this mere happenstance or a suggestion that all of the proteins in this series interact to form a complex? Although sucrose gradient centrifugation was developed precisely to ask this question, the negative result may merely have reflected the enormous dilution involved in these assays. New techniques of molecular crowding and cooperative enzyme kinetics will be employed to readdress this question.

- ANNUAL REPORT OF THE METABOLIC DISEASES BRANCH  
National Institute of Diabetes and  
Digestive and Kidney Diseases

The general goals of the Branch are to investigate the mechanism of action of hormones controlling ion transport and mineral metabolism and to investigate the immunological and pathological factors mediating kidney disorders. The branch currently includes sections of Mineral Metabolism (Dr. Marx), Endocrine Regulation (Dr. Aurbach) and Kidney Disease (Dr. Balow). Integration of these sections is related to common interests in the pathophysiology of metabolic disorders which interface with the kidney. Systems under study include renal and skeletal tissue, transgenic mice, isolated cells (kidney and parathyroid in culture, hormone receptors (beta adrenergic, parathyroid hormone, calcitonin and 1,25 dihydroxy vitamin D), parathyroid cell growth factors, and T cell and B cell function in disorders of immunoregulation.

#### Analysis of Hormone Receptor

Interactions with several hormone receptors regulating growth or adenylate cyclase are under study. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations, bovine parathyroid endothelial cells, rat osteosarcoma cells and rat liver membranes. Control of receptor biosynthesis in isolated cell culture systems is being studied with a view toward gaining knowledge about the molecular biology of receptors and how they are linked to intracellular response systems. A new method has been developed for immunocytology of cAMP and cGMP to study compartmentalization of signal transduction [ Drs. Barsony and Marx].

#### Primary Hyperparathyroidism and Familial Hypercalcemia

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case findings have produced approximately 85 kindreds for analysis. These studies allowed segregation of the most common familial variants into two apparently distinct disease syndromes - familial multiple endocrine neoplasia type 1 (FMEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from FMEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as

autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. FHH accounts for approximately 10% of all unsuccessful parathyroidectomies in hypercalcemia. In FHH the ionized and ultrafiltrable calcium concentration in serum are elevated in proportion to the increase in total calcium. In these patients the filtrable load of calcium is high in association with a marked decrease in renal calcium clearance. Even when these patients become surgically hypoparathyroid, the low renal clearance of calcium is strikingly persistent during calcium infusion. The concentration of parathyroid hormone in plasma is lower in patients with FHH than in typical primary hyperparathyroid patients with similar degrees of hypercalcemia whether assessed by PTH radioimmunoassay or by renal clearance of CAMP or phosphate. The parathyroid glands show hyperplasia in most cases. In several kindreds one or more members have exhibited life-threatening primary hyperparathyroidism in the neonatal period. This may result sometimes from a double dose of the FHH gene. Dispersed parathyroid cells from one severely affected neonate showed a striking decrease in sensitivity of PTH secretion to extracellular calcium. This disorder may reflect mutation in a gene that directs calcium recognition in both the parathyroid and renal tubular cell.

Familial multiple endocrine neoplasia type I (FMENI) is an autosomal dominant disorder characterized by hyperfunction of parathyroids, pancreatic islets, and anterior pituitary. Affected organs show features suggestive of increased proliferation. Virtually all subjects expressing the gene show primary hyperparathyroidism. Primary hyperparathyroidism is usually first recognizable between ages 20-40, and it shows a high recurrence rate after subtotal parathyroidectomy (approximately 50% after 10 years). We have evaluated multiple indices for use in screening in a very large kindred. We tested 221 members and newly identified 16 as carriers. Albumin-adjusted calcium and PTH were most useful; gastrin and prolactin analyses were not useful for screening but showed promise in followup of known carriers. Analysis in this family has revealed linkage to a locus on the long arm of chromosome 11. The MEN1 gene is a growth suppressor gene like the retinoblastoma gene; MEN1 related tumors are being screened for loss of heterozygosity at this locus. Such loss of heterozygosity has established that most parathyroid tumors in FMEN1 are monoclonal. Tumors with small deletions could speed identification of the MEN1 gene. [Drs. Marx, S. Bale, A. Bale, Mulvihill, Sparkes, Brandi, Aurbach, Sakaguchi, Friedman, Spiegel].

With cultured bovine parathyroid cells, we found abnormally high mitogenic activity in plasma from 23 of 27 subjects with FMEN1. Well-characterized growth factors or known parathyroid secretagogues showed far less parathyroid mitogenic activity than these FMEN1 plasmas. The mitogenic factor(s) appear to be a protein of 14,000 mw. We have begun purifying this factor for

further characterization. We have obtained evidence that the factor is related to basic fibroblast growth factor. Analysis of plasmas from one large kindred with FMEN1 suggests that high parathyroid mitogenic activity precedes primary hyperparathyroidism and may begin at very early ages. [Drs. Zimering Brandi, Sakaguchi, Aurbach, Goldsmith, Marx].

Studies on noninvasive and invasive modes of localizing parathyroid tumors continue. Parathyroid adenoma localization has been evaluated using the new non-invasive magnetic resonance imaging technique. Initial results were disappointing but the acquisition of a specialized neck collar has led to better resolution in the paratracheal and mediastinal areas. Patients are currently under evaluation with this new technique. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 450 of cases tested, the identification of abnormal masses of tissue proven at surgery to be parathyroid. In the most difficult cases, localization of parathyroid tissue can be aided by identifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Fine needle aspiration is another new method that can obviate other invasive localization procedures. We have aspirated with guidance by computerized tomography or ultrasound approximately 20 such lesions that were subsequently confirmed surgically as parathyroid. RIA of the aspirates showed high concentrations of PTH in all but one. Eight mediastinal adenomas have been treated nonsurgically by percutaneous injection via catheter of occlusive agents into the arterial blood supply with 7 complete and one partial remissions. [Drs. Aurbach, Marx, Spiegel, Zimering, Weinstein, Streeten, NIDDK: Dr. Norton, NCI, Drs. Doppman, Miller, and others, Diagnostic Radiology, CCI].

Rapid determination of intraoperative UcAMP excretion (using the Gammaflo machine for rapid cAMP radioimmunoassay) has proven to be a valuable tool in guiding surgery for primary hyperparathyroidism, particularly in patients with multigland disease. Persistent elevation of UcAMP requires continued search for abnormal tissue even after 1 or more abnormal glands have been removed. A rapid (mean 1.5 hours) drop in UcAMP to less than 50% of the baseline obviates the need for continued exploration even in cases where histologic confirmation of parathyroidectomy is lacking. Spurts in UcAMP above baseline may provide a clue to the location of abnormal parathyroid tissue. [Drs. Spiegel, Marx, Zimering, Weinstein, Streeten, and Aurbach, NIDDK: Dr. Norton, NCI Surgery].

Determination of urinary cAMP excretion postoperatively in patients undergoing neck exploration for primary hyperparathyroidism is a useful method for assessing postoperative parathyroid function. UcAMP excretion declines postoperatively in all patients in whom hypercalcemia is corrected but not in those with persistent hypercalcemia. In



patients becoming severely hypocalcemic (and requiring vitamin D therapy) postoperatively, UcAMP measurement enables one to distinguish patients with decreased parathyroid reserve as the cause for hypocalcemia (low UcAMP excretion) from patients with healing osteitis fibrosa ("hungry bones") with secondary hyperparathyroidism as the basis for hypocalcemia. UcAMP in the latter group is often elevated but can be suppressed if serum calcium is normalized. Elevated UcAMP excretion postoperatively in the face of hypocalcemia enables one to predict that vitamin D therapy will be required temporarily (if at all) and precludes the need for parathyroid autografts. [Drs. Spiegel, Marx, Zimring, Weinstein, Streeten, and Aurbach, NIDDK].

Postoperative patients with surgically corrected hyperparathyroidism are being actively evaluated in a five year follow up study [Dr. Udelsman, Norton NCI, Drs. Marx, NIDDK]. These patients are being studied for sequelae such as hypoparathyroidism, recurrent hyperparathyroidism, and complications such as vocal cord paralysis.

#### Secretion of Parathyroid Hormone

PTH secretion from parathyroid glands in vivo and cells in vitro is controlled by intracellular calcium and cyclic AMP. Control by calcium is altered in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Agents that alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP, but most of its effect to inhibit secretion is independent of changes in cellular cAMP.

Calcium inhibition of parathyroid hormone secretion is controlled through a complex set of mechanisms. We have shown previously that classical voltage, sensitive calcium channels are important in controlling parathyroid hormone secretion and that their action is mediated through a guanine nucleotide regulatory protein. It is also known that the growth of parathyroid cells is controlled by calcium. We have utilized a cloned rat parathyroid cell line (PT-r) to study this phenomena.

Proteoglycans synthesized by the PT-r cell have been characterized and effects of calcium on synthesis studied. One proteoglycan is located both intracellularly and on the external surface of the cell. Distribution of this proteoglycan between cell cytosol and cell surface is controlled by calcium. This effect on cell surface proteoglycans may be important in calcium regulation of growth as well as secretion. [Drs. Yanagishita, Sakaguchi, Brandi, and Aurbach].

We have recently shown that the PT-r cell produces acidic FGF (aFGF) and also bears receptors for aFGF. Calcium controls release of aFGF from the cell and also regulates synthesis of the aFGF receptor in this cell. These mechanisms may be important

mediators of calcium-controlled growth of the cell [Drs. Sakaguchi, Brandi, and Aurbach].

We have also cloned endothelial cells from bovine parathyroid tissue. These cells are distinct from the parathyroid epithelial cells that secrete hormone. As is characteristic of endothelial cells they contain Factor VIII-related antigen, take up acetylated low density lipoprotein and show ultrastructural features comparable to other endothelial cells. Using this cloned cell system we have shown that it is the target of antibodies developed in autoimmune hypoparathyroidism and also is the target for a parathyroid cell growth factor identified in multiple endocrine neoplasia type I. Classical  $H_2$  type histamine receptors have been identified by function (cAMP production) and ligand binding studies in these cells. [Drs. Brandi, Sakaguchi, Zimering, Falchetti, and Aurbach].

#### Vitamin D Resistance and Related Disorders

The role of  $1,25(OH)_2D_3$ , the most potent natural metabolite of vitamin D, has been assessed in hypocalcemic states. This very rapidly acting drug has simplified the management of hypocalcemia following parathyroidectomy: during this time skeletal remineralization imposes large but rapidly diminishing requirements for calcium.

We have evaluated patients with extreme resistance to  $1,25(OH)_2D$ . This can be a transient state as following parathyroidectomy or a permanent state as in familial cases. We have evaluated 20 patients with familial resistance to  $1,25(OH)_2D$ . Most patients have hypocalcemic rickets, usually with associated total alopecia. The alopecia is associated with the highest grades of resistance to  $1,25(OH)_2D$ , implicating calcitriol in physiology of the hair follicle. Mineral homeostasis is usually improved by treatments that sustain  $1,25(OH)_2D$  levels at 10-100 times normal. Intestinal response to  $1,25(OH)_2D$  can be documented repeatedly with a new stable isotope technique [Drs. Yergey, Viera, Marx].

Specific intracellular defects have been evaluated using cultured skin fibroblasts from these patients. With skin fibroblasts cultured from normals, a typical  $1,25(OH)_2D$ -receptor can be identified by binding in soluble extracts, by nuclear uptake of hormone with intact cells, or by elution of occupied receptor from DNA-cellulose. Fibroblasts from patients with familial resistance to  $1,25(OH)_2D$  have shown a spectrum of defects including nonfunctional receptors, diminished numbers of receptors, and receptors with decreased hormone binding affinity. Among cases with normal hormone binding sites on the receptors some show receptors with deficient binding to nucleus while others show normal binding to nucleus but abnormal interaction with nonspecific DNA (as DNA-cellulose). Cellular action of  $1,25(OH)_2D_3$  can be analyzed by measuring its induction of the

25(OH)D 24-hydroxylase enzyme system. Cultured skin fibroblasts from all patients with hereditary resistance to 1,25(OH)<sub>2</sub>D exhibit defects in this induction. Immunocytochemistry reveals multiple rapid steps of reorganization of vitamin D receptors after calcitriol addition. Specific disruptions in these steps can be imaged in mutant cells from patients. [Drs. Marx, Barsony, MDE, NIDDK; Dr. Liberman, Israel; Drs. Pike (Baylor) and DeLuca (Madison)].

Fibroblast lines from patients with hereditary extreme resistance to 1,25(OH)<sub>2</sub>D<sub>3</sub> are being used to probe for normal functions of the 1,25(OH)<sub>2</sub>D<sub>3</sub> receptor. We have shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> can elevate intracellular cyclic GMP very rapidly (within 1-3 minutes). This response showed affinity and analog specificity characteristic of the 1,25(OH)<sub>2</sub>D<sub>3</sub> receptor and was absent in all "mutant" fibroblast lines although they retained a rapid cGMP response to nitroprusside and to androgens. Thus a 1,25(OH)<sub>2</sub>D<sub>3</sub> receptor mediates this rapid response. [Drs. Barsony, Marx].

## Studies of the pathogenesis of glomerulosclerosis

The renal cell biology unit is interested in the cellular mechanisms leading to glomerular scarring, the emphasis being on non-immunologically mediated diseases. Our general hypothesis is that abnormalities in the growth regulation of resident glomerular cells play a major role in the development of glomerulosclerosis. We have developed methods using both in vitro approaches with glomerular cell culture and in vivo techniques using transgenic mice, the newly developed nonobese diabetic mouse (NOD) and subtotal nephrectomy (renal ablation) in rats. L. Striker, G. Striker, T. Doi, E. Peten, S. Elliot, R. Perfetti and L. Agodoa)

### III. Glomerulosclerosis

#### A. In Vivo Studies.

1. SV40 transgenic mice: Mice transgenic for the early region of simian virus 40 develop proteinuria and progressive glomerulosclerosis. We have postulated that large T antigen causes abnormal glomerular cell proliferation which induces glomerulosclerosis after unilateral nephrectomy. We found that unilateral nephrectomy markedly increased the severity of the glomerulosclerotic lesions in female mice but not in males. The sclerosis was associated with a marked increase in the size of the glomerular profiles. (K. MacKay, L. Striker, G. Striker)

2. Mice transgenic for GH, GHRF and IGF-I: We found that mice transgenic for GH and GHRF develop severe progressive glomerulosclerosis with an increase in the size of the glomeruli. By contrast, in IGF-I transgenic mice, while the glomeruli are also moderately enlarged, they do not develop glomerulosclerosis. This suggests that overexpression of certain growth factors, such as growth hormone and growth hormone releasing factor, may have effects in addition to those on cell growth. These may be important in inducing those additional changes which result in glomerulosclerosis. The elucidation of the pathogenetic events in this model may lead to further understanding of the general pathogenesis of glomerulosclerotic lesions. (T. Doi, L. Striker, E. Peten, G. Striker)

3. Nonobese diabetic (NOD) mice: NOD mice develop autoimmune diabetes mellitus, which predominates in females and leads to death if untreated with insulin. We compared the glomerular size, morphology, composition of the glomerulosclerosis and urinary abnormalities in mice with and without overt diabetes. Hyperglycemia was rapidly followed by an increase in glomerular size, mesangial sclerosis and proteinuria. We found an increase in the amount of mRNA coding for collagen IV shortly after the occurrence of diabetes. These mice could provide a good model of nephropathy in a genetically determined

model of diabetes mellitus. This study suggests that hyperglycemia triggers rapid glomerular lesions, if the glomeruli have a genetic propensity to develop sclerosis. (T. Doi, L. Striker, G. Striker)

4. Kidney disease in diabetic Pima Indians. We have undertaken a retrospective study of the glomerular lesions occurring in diabetic Pima Indians. They have a very high frequency of NIDDM. We have developed morphometric methods to measure the glomerular surfaces and determine whether glomerulosclerosis is associated with hypertrophy. (L. Striker, P. Bennett, G. Striker, C. Pesce, K. Schmidt)

5. Ablation model. Subtotal nephrectomy in rats is the most extensively studied model of progressive glomerulosclerosis. We are analyzing the early cellular events that may lead to glomerular destruction and found that there is an increase in glomerular and arterial cell turnover which occurs weeks before there is any extracellular matrix deposition. (L. Striker, T. Doi, C. Pesce, G. Striker)

## B. In Vitro Studies.

1. Murine and human glomerular cells: We have developed lines of mouse epithelial, mesangial and endothelial cells from normal mice and have been investigating their response to growth peptides (IGF-I, TGF-beta) and insulin. We have also undertaken studies on the role of IGF-I in the cell cycle of human mesangial cells, and provided evidence that it is a progression factor. Finally, we have shown that mouse mesangial cells not only respond to, but also produce, IGF-I immunoreactive molecules and IGFs binding proteins. This suggests that these peptides may play an important role in glomerular function. (T. Doi, R. Perfetti, S. Elliot, L. Striker, G. Striker)

2. Endothelial cells and insulin: We have identified a receptor for insulin on the surface of a clone of endothelial cells from normal mice. (S. Elliot, F. Conti, L. Striker, G. Striker)

3. Development of lines of mesangial cells from mice that develop glomerulosclerosis: We have developed lines of mesangial cells from mice transgenic for growth hormone and from NOD mice. We are presently examining their cell cycle and their extracellular matrix synthesis. We are postulating that there are phenotypic changes in the resident cells from glomeruli which develop glomerulosclerosis. (L. Striker, E. Peten, R. Perfetti, S. Elliot, G. Striker)

## KIDNEY DISEASES

The Kidney Disease Section conducts research on normal cell biology of various components of the kidney and on clinical and experimental renal diseases. Lupus nephritis and membranous nephropathy are the primary subjects of study. Patients as well as animal models are used to develop insights into pathogenetic mechanisms and to test novel immunosuppressive drug therapies which might have salutary effects on the course of these nephropathies.

### I. Glomerulonephritis and lupus nephritis

A. Immunopathogenesis. Murine models are being utilized to investigate the different components of lupus nephritis. The modulating effects of cyclophosphamide on immune responses in normal mice and on the renal lesions of nephritic mice are being investigated. Studies of differences among the murine strains have provided new approaches to study of the diverse manifestations and response to treatment of human lupus nephritis. (Austin, Patel, Balow).

B. Immunoregulatory studies. Heightened and poorly regulated B lymphocyte activity is characteristic of systemic lupus erythematosus. Defective T cell activity is also commonly present. Studies are in progress to investigate abnormalities of signal transduction in B and T lymphocytes from patients with lupus, using membrane calcium flux and expression of various genes in activated cells which could account for pathogenic humoral and cellular immune reactions in lupus. (Tsokos, Boumpas, Yamada, Patel, Balow).

C. Proliferative lupus nephritis. Studies have shown that intermittent pulse cyclophosphamide therapy is superior to conventional prednisone in management of lupus nephritis. No direct comparisons of pulse corticosteroids and pulse cyclophosphamide have been performed. Patients with proliferative lupus nephritis are being treated with pulse methylprednisolone or pulse cyclophosphamide to compare these two types of drugs and to assess whether intensity or duration of cyclophosphamide therapy is more important in stabilizing the renal disease. Immunologic studies of changes in lymphoid cell function by the various drug regimens are being pursued to identify techniques which will maximize efficacy and to improve monitoring of drug treatment. (Balow, Austin, MacKay and members of ARB, NIAMS).

D. Membranous nephropathy. Membranous nephropathy is associated with substantial cardiovascular morbidity from nephrotic syndrome and causes an insidious loss of renal function in patients with lupus and in those patients with idiopathic forms of this disease. Preliminary evidence indicates that the immunopathogenesis of membranous nephropathy is distinct from that of most proliferative forms of glomerulonephritis. Current protocols involve examination of the pathophysiology and histopathology of the glomerular lesions in membranous nephropathy,

as well as evaluation of the comparative efficacy of prednisone, cyclophosphamide and cyclosporin A in patients idiopathic and lupus related forms of this renal disease. (Balow, Austin, MacKay).

E. Transforming growth factor and glomerular reactions. The mechanisms responsible for normal growth and for pathogenic cellular reactions within the glomerulus are poorly understood. The signal transducing agent, transforming growth factor-beta, has a complex interaction with glomerular cells. Studies are underway to characterize the nature of the receptors for this growth factor on glomerular cells. In addition, several parameters, such as proliferation, fibronectin secretion and proteoglycan synthesis, will be used to evaluate the responses of these cells to binding of transforming growth factor. (MacKay).

## II. Role of Complement in Glomerulonephritis.

A. Nephritic factors. Patients with membranoproliferative glomerulonephritis and lupus nephritis develop autoantibodies reactive with complement converting enzymes which leads to abnormal consumption of complement components. These nephritic factors may participate in the pathogenesis of the renal diseases. Epstein-Barr virus transformed and sustained B lymphocyte lines which actively produce nephritic factors have been produced. One line from a patient with membranoproliferative glomerulonephritis secretes an IgG antibody which binds and stabilizes the alternative pathway C3 convertase enzyme. Another from a patient with lupus binds the classic pathway C3 convertase. Anti-idiotypic antibodies to nephritic factors have been isolated. The characteristics of ligand binding, receptor turnover and modulation by the nephritic factors, and idiotypic binding sites are under study. (Tsokos, Schwartz, Patel, Balow).

B. Complement in immune regulation. Abnormal levels of complement components and deposition in sites of immunological reactions are characteristic of several forms of nephritis. The interactions of complement components and activation products with receptors on lymphoid cells are being studied to gain new insights into their potential role in lupus nephritis, membranoproliferative glomerulonephritis and other renal disorders. A deficiency in number or function of complement receptors on B lymphocyte may predispose to the appearance of autoantibodies associated with these diseases. Studies are underway to determine the mechanism of the modulation of B lymphocyte responses through interaction of the complement receptor with natural complement ligands, Epstein-Barr virus, and monoclonal antibodies to complement receptors on B cells. (Tsokos, Balow).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43002-25 MDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure, Secretion and Mechanism of Action of Parathyroid Hormone

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G.D. Aurbach

Chief, MDB, NIDDK

OTHERS: S. Doi, M.D.

Visiting Associate, MDB, NIDDK

M.L. Brandi, M.D., Ph.D.

Visiting Associate, MDB, NIDDK

K. Sakaguchi, M.D., Ph.D.

Visiting Associate, MDB, NIDDK

M. Zimering, M.D.

Medical Staff Fellow, MDB, NIDDK

Y. Fujii, M.D.

Visiting Fellow, MDB, NIDDK

## COOPERATING UNITS (if any)

Endocrine Unit, Massachusetts General Hospital  
Department of Medicine Yale University

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Endocrine Regulation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5

## PROFESSIONAL:

4

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is the purpose of this project to study the secretion, function, and mechanism of action of parathyroid hormone, its relationship to human disease, and to develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine, rat and human parathyroid hormone have been determined. Synthetic polypeptides representing bovine rat and human parathyroid hormone have been synthesized. These molecules show all the biological properties of the native hormonal polypeptides. Highly sensitive radioimmunoassays for the hormone have been developed and are being modified further for improved clinical diagnostic parameters. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. Isolated parathyroid cells and culture systems have been developed that allow studies on secretory control of parathyroid hormone, and provide test systems to elucidate the pathophysiology of certain hypoparathyroid and hyperparathyroid states.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43003-25 MDB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mode of Action of Thyrocalcitonin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	J. Barsony, M.D.	Visiting Associate	MDB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.1

PROFESSIONAL

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It will also be of interest to solubilize the receptors and characterize them chemically. Calcitonin increases cAMP in MCF 7 breast cancer cells. At 300-fold lower concentration calcitonin decreases cAMP in these cells. The decrease in cAMP is prevented by preexposure of cells to agents that interfere with inhibitory guanyl regulatory proteins. Intracellular compartmentalization of cAMP accumulation after calcitonin has been imaged after microwave fixation of cells. The cAMP accumulates initially along the plasma membrane but within 1 to 3 minutes accumulates much closer to the nucleus.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43006-15 MDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Hyperparathyroidism: Etiology, Diagnosis and Treatment

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G.D. Aurbach

Chief, MDB, NIDDK

OTHERS: S.J. Marx, MD.  
E. Friedman, M.D.  
J. Merendino, M.D.  
M.L. Brandi, M.D., Ph.D.  
K. Sakaguchi, M.D., Ph.D.  
M. Zimering, M.D.  
E. Streeten, M.D.

Chief, Min. Metab. Sec., MDB, NIDDK  
Medical Staff Fellow, MPB, NIDDK  
Medical Staff Fellow, MPB, NIDDK  
Visiting Associate, MDB, NIDDK  
Visiting Associate, MDB, NIDDK  
Medical Staff Fellow, MDB, NIDDK  
Senior Staff Fellow, MDB, NIDDK

## COOPERATING UNITS (if any)

Radiology Department, CC; Surgery Branch, NCI; Digestive Diseases Branch, NIDDK

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Endocrine Regulation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS

4.75

## PROFESSIONAL

2.50

## OTHER:

2.25

## CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent or recurrent hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house clinical studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, ultrasonography, radiothallium scanning, magnetic resonance imaging, CAT scanning, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction. In vitro evaluation of parathyroid and other endocrine tissue involves tissue culture, chemistry and determination of linkage with DNA or RNA probes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43008-09 MDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Vitamin D Resistance and Related Disorders

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.J. Marx, M.D. Chief, Min. Metab. Sec. MDB, NIDDK

Others: J. Barsony, M.D. Visiting Associate MDB, NIDDK  
 W. McKoy Chemist MDB, NIDDK  
 L. De Marco, M.D., Ph.D. Special Volunteer MDB, NIDDK  
 C. Smith, Ph.D. IRTA MDB, NIDDK  
 L. Kohidai, M.D. Fogarty Fellow DB, NIDDK

## COOPERATING UNITS (if any)

Metabolism Unit, Beilinson Hospital, Petah Tiva, Israel (U. Liberman)  
 Cell Biology Department, Baylor University (J.W. Pike)  
 Biochemistry Department, University of Wisconsin, Madison (H.F. DeLuca)  
 Hormone Action and Oncogenesis Section, NCI (G. Hager)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Mineral Metabolism Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

4.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The calciferols were the first class of hormonally active steroids to be discovered and also the first for which subjects with hormone resistance could be identified. With recognition that vitamin D is the precursor for 1,25-dihydroxyvitamin D, it has become possible to characterize defects in the activation (1-hydroxylation) of vitamin D and defects in the target action of activated (1,25-dihydroxy)vitamin D. We have demonstrated a broad spectrum of manifestations of hereditary resistance to 1,25(OH)2D ranging from infantile rickets with alopecia and no intestinal response to calciferols to adult onset osteomalacia with satisfactory intestinal response to high doses of calciferols and with no epidermal abnormalities. This syndrome usually results from a mutation in the gene for the vitamin D receptor. Cultured skin fibroblasts display many components of the 1,25(OH)2D effector system. Skin fibroblasts from all subjects with hereditary resistance to 1,25(OH)2D display abnormalities in this effector system, and defects in many discrete steps of this pathway have been identified with these cells. Cells with mutations in the 1,25(OH)2D effector pathway can be used to explore mechanisms of calciferol action. They have been used to establish that the 1,25(OH)2D receptor mediates an extremely rapid (1-3 minutes) rise of cyclic GMP in response to 1,25(OH)2D3 and that certain receptor mutations compromise many receptor functions but allow another function to be retained normally. This establishes that 1,25(OH)2D receptors couple to different responses by distinct mechanisms.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43009-05 MDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Mineral Metabolism

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	J.J. Mulvihill, M.D.	Chief, Clin. Genetics Sec.	CEB, NIDDK
	S.J. Bale, Ph.D.	Senior Staff Fellow	EEB, NCI
	M.L. Brandi, M.D.	Visiting Associate	MDB, NIDDK
	W. McKoy	Chemist	MDB, NIDDK
	G. Aurbach, M.D.	Chief	MDB, NIDDK
	E. Streeten, M.D.	Senior Staff Fellow	MDB, NIDDK
	E. Friedman, M.D.	Visiting Fellow	MPB, NIDDK
	A. Spiegel, M.D.	Chief	MPB, NIDDK
	J. Norton, M.D.	Head, Metabolism Sec.	SB, NCI

## COOPERATING UNITS (if any)

EEB, CEB, LB, NCI, MPB

Belvedere Medical Center - Carlisle, PA (J. Green)

Genetics Department - Yale University (A. Bale)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Mineral Metabolism Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.7

## PROFESSIONAL

1.5

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Disorders of mineral metabolism have been evaluated with methods extending from epidemiology to cellular and molecular biology. Two forms of familial hyperparathyroidism have been characterized in detail. Familial hypocalciuric hypercalcemia is an autosomal dominant trait associated with abnormal interactions with calcium in parathyroid and kidney. Familial multiple endocrine neoplasia type 1 (FMEN1) is an autosomal dominant trait causing hyperfunction of parathyroids, pancreatic islet and anterior pituitary. It is associated with gradual but abnormal proliferation of the tissues affected. Genetic linkage studies in a large kindred have localized the MEN1 gene to the long arm of chromosome 11. Plasma from affected persons shows high mitogenic activity upon cultured bovine parathyroid cells. This mitogenic activity in plasma may contribute to primary hyperparathyroidism in FMEN1. Analysis of blood and parathyroid tumor DNA has revealed that FMEN1 parathyroids often show clonal loss of alleles in the region of the FMEN1 gene on chromosome 11. Thus the FMEN1 gene functions as a tumor suppressor gene, analogous to the retinoblastoma gene. Analysis of sporadic parathyroid adenomas revealed that 25% showed allelic loss in a similar region. Thus the clonal inactivation of the FMEN1 gene may also be a contributing factor in many sporadic parathyroid adenomas.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43200-11 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Disorders of Immune Regulation in Patients with Systemic Lupus Erythematosus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.:	G. C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	D. T. Boumpas	Visiting Associate	MDB, NIDDK
	H. Yamada	Visiting Fellow	MDB, NIDDK
	A. Patel	Biologist	MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Disease Branch

## SECTION

Kidney Disease Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.00

## PROFESSIONAL:

2.00

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with systemic lupus erythematosus have been found to have various disturbances of the cell-mediated immune response. Cellular aberrations include enhanced spontaneous B lymphocyte activity with abnormal triggering in vitro, deficient immunoregulatory T lymphocyte circuits, deficient cytotoxic responses, including natural killer cell activity, alloantigen and viral cytotoxicity, and abnormal production of and response to different lymphokines as well as increased expression of proto-oncogenes in highly activated peripheral blood lymphocytes.

CD4+ and CD4/CD8+ T lymphocyte receptor cells and cell lines from patients with active lupus nephritis provide help to autologous B lymphocytes to produce nephritogenic antibodies. The goal of these studies is to further elucidate the mechanisms of these alterations of the immune system which are apparently involved in the pathogenesis of this disease.

The modulation of the above disturbances by immunosuppressive agents, i.e. corticosteroids and cyclophosphamide, is actively studied, aiming at the restoration of normal immune status in these patients.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43201-06 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production and Characterization of Nephritic Factors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and instituta affiliation)

P. I.:	G. C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	R. Schwartz	Guest Researcher	MDB, NIDDK
	A. Patel	Biologist	MDB, NIDDK

## COOPERATING UNITS (if any)

SUNY Medical Center, Syracuse, NY (Dr. R. Spitzer)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Kidney Disease Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.00

## PROFESSIONAL:

1.00

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The nephritic factor (NeF) of the alternative pathway of complement has been found in the sera of patients with membranoproliferative glomerulonephritis (MPGN) and partial lipodystrophy and has been described as a factor which is able to induce cleavage of the third component of complement (C3) in normal human serum through the alternative pathway. It has been demonstrated that NeF binds to and stabilizes C3bBb (alternative C3 convertase). NeF appears to be antigenically and structurally similar to IgG and therefore it might be an autoantibody directed against C3bBb complex. The relation between the development of renal lesions and the NeF mediated persistent hypocomplementemia remains unexplained. B lymphocytes from patients with MPGN were used to establish cell lines secreting NeF of either IgG or IgM classes. Sera of patients with MPGN were found contain anti-idiotypic antibodies to NeF. We isolated 3 different anti-idiotypic antibodies and found that monoclonal and several polyclonal NeF share at least one idiotope. To verify this observation we are in the process of repeating these experiments using hetero-anti-idiotypic antibodies. Nucleotide sequencing of different NeF will answer the question whether NeF are direct products of germline genes or have undergone mutations as a result of antigenic stimulation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43202-07 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Human Immune Response by Complement

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.:	G. C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	E. D. Anastassiou	IRTA Fellow	MDB, NIDDK
	H. Yamada	Visiting Fellow	MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Kidney Disease Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.50

## PROFESSIONAL:

0.50

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Complement factors and breakdown products acting through cell surface membrane receptors block the differentiation of human B lymphocytes into immunoglobulin secreting cells. Complement receptors are associated with B cell surface immunoglobulin under certain circumstances. Furthermore, complement receptor expression is cell-cycle dependent.

Monovalent ligands inhibit while polyvalent enhance the anti-IgM induced human B cell increase in intracytoplasmic calcium ion concentration and cell proliferation.

Understanding of the mechanism of regulation of immune responses by complement and the role of complement receptors on human B cells is crucial for the understanding of the immunopathogenesis of autoimmune diseases since they are frequently associated with complement activation, depression of complement factor levels and changes in complement receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43204-10 MDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunosuppressive Drug Therapy in Lupus Glomerulonephritis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. E. Balow

Senior Investigator

MDB, NIDDK

Others: H. A. Austin

Medical Officer

MDB, NIDDK

COOPERATING UNITS (if any)

NIAMS (J. H. Klippel, P. H. Plotz, A. D. Steinberg, R. Wilder).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.50

PROFESSIONAL:

1.50

OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The optimal treatment of the proliferative forms of kidney disease associated with systemic lupus erythematosus is controversial. The efficacy of intensive, intermittent immunosuppressive drug therapy is being evaluated in patients with active lupus glomerulonephritis. A comparison is being made between intermittent pulse doses of corticosteroid and cyclophosphamide, as well as between a short and long course of pulse cyclophosphamide. Patients with renal biopsy documented active glomerulonephritis are treated with prednisone and randomized to receive concomitantly (a) intravenous pulse methylprednisolone monthly for 6 months, or (b) intravenous pulse cyclophosphamide monthly for 6 months, or (c) pulse cyclophosphamide monthly for 6 months followed by a maintenance regimen of pulse cyclophosphamide every 3 months for an additional two years. During the final 24 months of the study, all patients continue to receive low dose, alternate day prednisone. Active disease, as manifested by renal functional deterioration, increased proteinuria or worsened urinary sediment, is treated by increased prednisone. Comparison will be made of the number of favorable outcomes of renal function, glomerular pathology and drug related toxicities occurring in each treatment.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43205-13 MDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Biopsy Pathology in Systemic Lupus Erythematosus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. E. Balow  
Others: H. A. Austin

Senior Investigator  
Medical Officer

MDB, NIDDK  
MDB, NIDDK

COOPERATING UNITS (if any)

Armed Forces Institute of Pathology, Washington, DC  
(T. Antonovych, S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.25

PROFESSIONAL:

0.25

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Diverse pathogenetic factors are operant in systemic lupus erythematosus and lead to different forms of lupus nephritis. Detailed analysis of renal biopsy pathology is being conducted on specimens from patients with systemic lupus erythematosus.

Biopsies are classified by standard major category of lupus nephritis, as well as scored on a semiquantitative scale for specific histologic changes which indicates the extent and severity of active inflammatory lesions and of chronic atrophic, fibrosing and sclerosing features. The patterns of immune complex deposition and lymphoid cell interaction with different segments of the nephron are being investigated by immunohistologic techniques and electron microscopy. Computer-based morphometric techniques are being developed to analyze more precisely the changes in renal pathology during the course of lupus nephritis.

These approaches have facilitated the analysis of the effects of various types of immunosuppressive agents used to halt the progression of lupus nephritis and they will enhance our understanding of the pathogenesis of the kidney disease of systemic lupus erythematosus.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43210-06 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Disease in Transgenic Mice

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	K. MacKay	Medical Staff Fellow	MDB, NIDDK

## COOPERATING UNITS (if any)

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We previously described the development of glomerulosclerosis in mice transgenic for large T-antigen, a gene whose in vitro expression markedly increases proliferation of cultured cells. In the current study we sought to determine the effect of unilateral nephrectomy on these sclerosis-prone animals which have a genetically defined potential for increased renal growth. In comparison with sham nephrectomy animals, nephrectomized transgenic female mice had significantly larger kidneys, larger glomeruli, more cells per glomerulus and more severe glomerulosclerosis. Nephrectomized transgenic male animals had variable increases in kidney size, no significant increase in glomerular size or cellularity, and no worsening of glomerulosclerosis. In non-transgenic female animals nephrectomy induced an increase in kidney size but not in glomerular size and failed to induce glomerular lesions. A close correlation was found between glomerular size and severity of glomerulosclerosis in these animals.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43211-06 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histopathology of Renal Lesions in Pima Indians

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	C. Pesce	Visiting Associate	DCBD, NCI
	K. Schmidt	Medical Staff	
		Fellow	DCBD, NCI

## COOPERATING UNITS (if any)

Epidemiology and Clinical Research Branch, NIDDK, Phoenix, Arizona  
(P. Bennett).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.7

## PROFESSIONAL:

1.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The weighed mean of the area and a size-class distribution of the glomeruli were calculated with a computer-assisted planimeter in a series of autopsy kidney specimens in Pima Indians affected by diabetes mellitus (DM) and in non-diabetic Pima Indians. These morphometric variables were also related to the severity of histologic glomerular lesions, graded on a four class scale. Glomerular size did not differ in the two groups, nor were differences in the size distribution discernible among the classes of histologic lesion. A similar pattern is found in the Causcasian population affected by type-II DM, whereas type-I DM is associated with an early phase of increase and a late phase of decrease in glomerular size. The glomeruli of diabetic Pima Indians did not decrease in size even at the stage of diffuse sclerosis, a finding that contrasts with what is commonly seen in vascular nephropathy. The NIDDK epidemiologic survey of Pima Indians allows recognition of DM early after onset and long-term follow up of the disease and its nephropathy. This offers a unique opportunity to study the natural history of diabetic nephropathy in a population with an incidence of type-II DM approaching 50% of the adults and an unusually young age of onset of the disease.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 BK 43217-06 MDB

## PERIOD COVERED

October 1, 1989 through May 31, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Lesions in Leukemias, Lymphomas and Carcinomas

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK

## COOPERATING UNITS (if any)

National Cancer Institute, Bethesda, Maryland (M. Merino, W. Travis).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We evaluated glomerular lesions in kidneys from patients who underwent nephrectomy for renal cancer. In areas non-invaded by the tumor there was, in one-half of the cases examined, marked mesangial proliferation and occasional synechiae. This suggested that the glomerular lesions we found could be mediated by growth factors released from the tumor.

In a separate group of patients we examined the effect of interleukin II on the kidneys of 19 patients who died in the Clinical Center. The occurrence of lesions was correlated with the creatinine and BUN level.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43220-05 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Expression of Angiotensin Converting Enzyme in Renal Glomeruli

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Striker Expert MDB, NIDDK

## COOPERATING UNITS (if any)

National Institute of Mental Health, Bethesda, Maryland (B. Martin);  
Emory University, Atlanta, Georgia (K. Bernstein).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43221-05 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Insulin REceptors in Glomerular Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. Elliot	Biologist	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	L. Striker	Expert	MDB, NIDDK

## COOPERATING UNITS (if any)

## LABORATORY

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews  
☐ (b) Human tissues  
☒ (c) Neither

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Insulin receptors were identified on glomerular endothelial cells. Insulin binding was time, temperature, and pH dependent and total specific binding was  $2.5 \pm 0.3\%/10^6$  cells. Binding analysis according to Scatchard resulted in a curvilinear plot. The  $K_d$  of the high affinity site was estimated to be  $1 \times 10^{-10}$ . Down-regulation of the insulin receptor was time and concentration dependent. The receptor, visualized with SDS-PAGE and autoradiography following crosslinking, contained an  $\alpha$  subunit of  $M_r$  of 125,000 daltons, similar to that of other tissues. Occupation of the receptor by insulin stimulated phosphorylation of the tyrosine kinase on the  $\beta$  subunit,  $M_r$  95,000 daltons, of the insulin receptor. Insulin stimulated glucose and amino acid uptake and was able to act as a weak progression factor. Therefore it was concluded that insulin is necessary for the metabolic function of glomerular endothelial cells, but not essential for DNA synthesis. It is conceivable that the abnormalities of the glomerulus in diabetes could be due to a dysfunctional endothelium, not able to perform its normal role in the glomerulus when presented with abnormal amounts of insulin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43222-05 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Murine Lupus Nephritis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	D. T. Boumpas	Visiting Associate	MDB, NIDDK
	A. D. Patel	Biologist	MDB, NIDDK

## COOPERATING UNITS (if any)

Armed Forces Institute of Pathology; Washington, DC (Drs. T. Antonovych and S. Sabnis).

## LAB/BRANCH

Metabolic Disease Branch

## SECTION

Kidney Disease Branch

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.75

## PROFESSIONAL:

0.75

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |                                      |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |                                      |
| <input type="checkbox"/> (a2) Interviews    |  |                                      |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Investigations of the pathogenesis and treatment of lupus nephritis are facilitated by the availability of inbred strains of mice that develop disease similar to human systemic lupus erythematosus. The natural evolution of the diverse histologic features of murine lupus nephritis is being studied to delineate the types of glomerular and tubulointerstitial lesions. Monoclonal antibodies and immunoperoxidase staining of frozen sections are employed to study the types and distribution of immunoglobulin deposited and the characteristics of the lymphoid cells in glomerular, vascular and tubulointerstitial lesions. The impact of biologic response modifiers on immunologic features is being investigated. The goal is to develop a model of a flare of lupus nephritis which would facilitate further investigations of immunopathogenic mechanisms. Innovative treatment strategies will be studied to refine our approach to this disease. Clinical, histologic and immunologic outcome parameters will be evaluated including detailed studies of renal morphology, the characteristics of peripheral blood lymphocytes and splenocytes employing flow cytometry, measures of immunoglobulin gene expression, and in vitro assays of alterations in humoral and cell mediated immune regulation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43224-04 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membranous Lupus Nephropathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H. A. Austin

Medical Officer

MDB, NIDDK

Others: J. E. Balow

Senior Investigator

MDB, NIDDK

K. MacKay

Expert

MDB, NIDDK

## COOPERATING UNITS (if any)

CC (E. Vaughan); NIAMS (J. Klippel); Stanford University; Stanford, CA (B. Myers). Armed Forces Institute of Pathology, Washington, D.C. (Drs T. Antonovych and S. Sabnis).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Kidney Disease Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.25

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☒ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

It is currently unknown whether therapeutic intervention will alter the course of membranous lupus nephropathy. In the present study, the efficacy and toxicity of three immunosuppressive drug regimens administered over a 12 month period will be evaluated in patients with membranous lupus nephropathy. Detailed tests of renal function (including radiolabelled compounds for glomerular filtration and renal plasma flow rates), glomerular permselectivity (using fractional clearance of graded dextrans) and kidney biopsy morphology will be performed at the beginning and end of treatment. Patients with systemic lupus erythematosus, nephrotic range proteinuria and biopsy documented membranous nephropathy will be randomized to receive: a) alternate day prednisone alone (control group), b) alternate day prednisone plus intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) alternate day prednisone plus oral cyclosporin A up to 200 mg per square meter body surface area daily. Lupus disease activity, renal function tests and drug toxicities will be monitored closely. Analysis will include comparison of the numbers of favorable outcomes of glomerular filtration rate, renal plasma flow, permselectivity, glomerular pathology and drug-related toxicities appearing in each treatment group.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 43225-03 MDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Changes Due to GH and IGF-I

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Striker Expert MDB, NIDDK

Others: T. Doi Visiting Scientist MDB, NIDDK  
L. Agodoa Medical Officer MDB, NIDDK  
E. Peten Special Volunteer MDB, NIDDK

COOPERATING UNITS (if any)

University of Washington, Seattle, Washington (R. Palmiter); School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.6

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Increased glomerular size occurs in the presence of normal maturation following unilateral nephrectomy in humans and animals and in disease states such as diabetes mellitus. The glomeruli are morphologically and functionally normal following nephrectomy in rats unless the remaining renal mass is severely reduced, in which case progressive glomerulosclerosis ensues. The hormonal regulation of compensatory hypertrophy is not fully understood, however total kidney IGF-I mRNA levels are increased following unilateral nephrectomy. This suggests a role for this hormone in hypertrophy of the adult kidney as well as in normal development. There are abnormalities in the circulating levels of GH in some diseases associated with increases in glomerular extracellular matrix and cell number such as diabetes mellitus. The availability of transgenic mouse strains expressing elevated levels of IGF-I, GH, and GHRF provides and opportunity to study the renal effects of chronic hormone exposure. We have observed that mice containing an MT-I IGF-I fusion gene develop large glomeruli which are normal in appearance, whereas those transgenic for either growth hormone or growth hormone releasing factor have large glomeruli which are hypercellular, whereas progressive glomerulosclerosis develops later.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43226-03 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of IGF-B on Glomerular Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. MacKay	Medical Staff Fellow	MDB, NIDDK
Others:	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK
	T. Doi	Visiting Scientist	MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |                                      |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |                                      |
| <input type="checkbox"/> (a2) Interviews    |  |                                      |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43227-03 MDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of IGF-I in the Biology of Mouse Glomerular Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	G. Striker	Director	DKUHD, NIDDK
Others:	T. Doi	Visiting Scientist	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK
	L. Striker	Expert	MDB, NIDDK

COOPERATING UNITS (if any)

Diabetes Branch, NIDDK (M. Lesniak, J. Roth).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The renal glomerulus is both a site of action and synthesis of IGF-I. We previously demonstrated the presence of IGF-I receptor and synthesis in glomerular mesangial cells. In this study we investigated the presence of specific IGF-I receptors on mouse glomerular endothelial and epithelial cells in culture. [<sup>125</sup>I]IGF-I specifically bound to the cell surface of both cell types. Maximum specific binding, 0.141 B/F for endothelial cells and 0.301 B/F for epithelial cells, was obtained at 22°C after 150 min incubation. The estimated K<sub>d</sub> values were 2.25x 10<sup>-9</sup> for endothelial cells and 1.5x10<sup>-9</sup> for epithelial cells. Cross-linking studies showed a single band of radioactivity with an estimated mol wt of 145K, consistent with the α-subunit of the IGF-I receptor. Radiolabelled IGF-I was not degraded by either cell types. these finding suggest a possible paracrine action of IGF-I in the renal glomerulus.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43228-03 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Human Glomerular Mesangial Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	T. Doi	Visiting Scientist	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project is inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43229-02 MDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Binding and Uptake of Mouse IgA by Mouse Glomerular Mesangial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Agodoa Medical Officer MDB, NIDDK

Others: S. Elliot Biologist MDB, NIDDK  
L. Striker Expert MDB, NIDDK

COOPERATING UNITS (If any)

Brown University, Providence, Rhode Island (R. Rifai).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43230-02 MDB

PERIOD COVERED

October 1, 1989 through December 31, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Proteoglycan Production by Mouse Glomerular Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	G. Striker	Director	DKUHD, NIDDK
Others:	K. MacKay	Medical Staff	
		Fellow	MDB, NIDDK
	L. Striker	Expert	MDB, NIDDK

COOPERATING UNITS (if any)

Department of Cell Biology, Yale School of Medicine, New Haven, Connecticut  
(J. Stow, M. Farquhar).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mouse glomerular epithelial cells previously characterized have been investigated for biosynthesis of proteoglycans. Confluent monolayers of epithelial cells were radiolabeled. It was found that these cells produced Heparan Sulfate basement membrane in high amounts. Analysis of immunoprecipitates showed both a mature proteoglycan and a precursor core protein band. These proteoglycans are an important determinant of the permselectivity properties of the glomerular basement membrane.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43231-02 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Idiopathic Membranous Nephropathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. : H. A. Austin Medical Officer MDB, NIDDK  
Others: J. E. Balow Senior Investigator MDB, NIDDK  
K. MacKay Expert MDB, NIDDK

## COOPERATING UNITS (if any)

Stanford University, Stanford, CA (Dr. B. Myers);  
CC (E. Vaughan, Nursing); Armed Forces Institute of Pathology,  
Washington, DC (Drs. T. Antonovych and S. Sabnis).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Kidney Disease Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.00

## PROFESSIONAL:

1.00

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with idiopathic membranous nephropathy are being studied to evaluate the efficacy and toxicities of the addition of intermittent cyclophosphamide or daily oral cyclosporin A to alternate day oral corticosteroid therapy. Efficacy will be judged by determinations of effective renal plasma flow, glomerular filtration rate and glomerular capillary wall permselectivity performed with dextran and urine protein (albumin and immunoglobulin) clearance techniques. Kidney biopsy morphology (including morphometric analysis) will be examined at the beginning and at the end of treatment as part of detailed studies of structure-function relationships and the efficacy of various therapeutic modalities.

Patients with membranous nephropathy and 2 or more grams per day of proteinuria will be treated with alternate day prednisone and will be randomized to receive: a) no additional therapy (control group), b) intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) oral cyclosporin A up to 200 mg per square meter body surface area daily for a total of 11 months. Analysis will include comparison of the number of favorable outcomes of glomerular function and pathology as well as drug-related toxicities observed in each treatment group at the end of the 12 months of study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43232-01 MD B

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Lesions in the Ablation Model: Role of Growth Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Co-PI:	G. Striker	Director	DKUHD, NIDDK
Others:	T. Doi	Visiting Scientist	MDB, NIDDK
	C. Pesce	Visiting Associate	DCBD, NCI

COOPERATING UNITS (if any)

Washington University, St. Louis, Missouri (S. Klahr, M. Purkenson);  
Department of Pathology, Kyoto University, Japan (H. Yoshida).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.9

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A subtotal nephrectomy in rats, the so called ablation model leads to a progressive destruction of the kidney with manifestations of chronic renal failure. We have postulated that the progressive glomerular lesions were due to an early increase in the turnover of glomerular resident cells. This leads to an abnormal glomerular growth, with an increase in the glomerular volume detectable using morphometric measurements. We have performed autoradiographies with 3H Thymidine and found that as early as two days following the subtotal nephrectomy there was an increase in the glomerular mitotic index as well as an increase in the turnover of the cells forming the arterial wall. These findings suggest that a dysregulation of cell growth is an early event in the development of glomerulosclerosis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 43233-01 MDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of IGF-1 Binding Proteins by Mouse Mesangial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Co-PI:	G. Striker	Director	DKUHD, NIDDK
Others:	R. Perfetti	Visiting Fellow	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK

## COOPERATING UNITS (if any)

Molecular, Cellular and Nutritional Endocrinology Branch, NIDDK (M. Rechler).

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology Unit

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We examined whether mesangial cells from normal mice synthesized IGF-I binding proteins in vitro. The supernatant from mesangial cells contained a single species of binding protein with a molecular weight of 30 KD. This protein was not glycosylated. It was found to be released in much higher amounts in sparse cells than in confluent cells. It was also found that the cells expressed mRNA for this binding protein, known as BP2. The binding protein release was coordinately regulated with the production of IGF-I by the cells. This new factor could participate in the control of mesangial proliferation and diseases associated with an increase in glomerular cell turnover could be partly due to a dysregulation in IGF-I-BP production and release.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43234-01 MDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interactions between TGF- $\beta$  and Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: K. MacKay

Expert

MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.75

PROFESSIONAL:

0.75

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Increases in glomerular cellularity and accumulation of extracellular matrix material and are prominent histologic findings in a number of clinical and experimental glomerular diseases. Transforming growth factor-beta (TGF- $\beta$ ) has been identified as a potentially important modulator of glomerular pathology based on its demonstrated ability to regulate proliferation and extracellular matrix synthesis by cultured glomerular cells. The goal of these studies is to better understand the expression, actions, and mechanisms of action of TGF- $\beta$  on glomeruli in vivo. We found that normal rat glomeruli contain high concentrations of TGF- $\beta$ 1, TGF- $\beta$ 2 and their corresponding mRNAs. We also found that a marked disparity exists between the TGF- $\beta$  receptor phenotype identified on cultured glomerular cells and the TGF- $\beta$  binding proteins or receptors which are present in intact glomeruli. Cultured glomerular cells express typical type I and II TGF- $\beta$  receptors. These receptors are not evident in intact glomeruli. Instead, glomeruli contain a unique group of disulfide linked TGF- $\beta$  binding proteins which bind TGF- $\beta$ 1 but not TGF- $\beta$ 2. These results suggest that glomeruli may respond quite differently than cultured cells do to TGF- $\beta$ . This could be due to pre-existing maximal stimulation of glomerular cells by endogenous TGF- $\beta$ , absence of typical cellular receptors for TGF- $\beta$ , or to modulation of response by the TGF- $\beta$ 1 selective binding proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 BK 43231-01 MDB

PERIOD COVERED

October=1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Lesions in Non-obese Diabetic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	T. Doi	Visiting Scientist	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK

COOPERATING UNITS (if any)

Joslin Diabetic Center, Boston, Massachusetts (M. Hattori); Department of Pathology, Kyoto University, Japan (H. Yoshida).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

NOD mouse spontaneously developed insulin dependent diabetes mellitus (IDDM) secondary to islet beta cell destruction. Shortly after the appearance of diabetes, NOD mice developed renal lesions which consisted of diffuse mesangial sclerosis, thickening of glomerular basement membrane and the development of albuminuria. These lesions closely mimic those of IDDM in humans. Morphometrical analysis showed that the kidney weight and glomerular size were increased in diabetic mice compared to non-diabetic mice. The glomerular volume/kidney weight ratio was also elevated in diabetic mice. These findings suggest that a discoordinate increase of glomerular size may play an important role for the development of diabetic nephropathy.

Dr. Hans J. Cahnmann, who joined the Clinical Endocrinology Branch in 1955 and has continued his laboratory work since 1975 as Scientist Emeritus, was a guest of the Protein Research Foundation, Osaka, Japan, from November 7 to 27, 1989. On November 24, Dr. Cahnmann delivered a lecture at the Institute entitled, "Thirty years of Thyroid Research". His host was Professor Tetsuo Shiba, who was a Visiting Fellow with Dr. Cahnmann from 1960 to 1962 and is currently at NIH as a Fogarty Scholar. Dr. Shiba was an organizer of the celebration of the 100th anniversary of NIH in Japan during 1987.

Dr. Bhabatarak Bhattacharyya, a Visiting Scientist from Calcutta, India, with Dr. Jan Wolff, was awarded the prestigious Bhatnagar Prize of the Indian government on March 28, 1990. Dr. Bhattacharyya was a Visiting Fellow with Dr. Wolff from 1972 to 1975, at which time he began his work on the chemistry of microtubules, for which the award was given. This is the third extended visit of Dr. Bhattacharyya to the Clinical Endocrinology Branch to continue his research in Dr. Wolff's laboratory.

On the occasion of the death of Prof. Serge Lissitzky of the University of Marseilles, one of the world's leading thyroidologists, Dr. Robbins was invited to contribute to a memorial issue of Biochimie in Prof. Lissitzky's honor. The paper entitled, "Binding of thyroxine to human plasma low density lipoprotein through specific interaction with apolipoprotein B (apoB-100)", by Salvatore Benvenia (Visiting Associate from Messina, Italy), Hans Cahnmann, Richard Gregg (NHLBI) and Dr. Robbins was published in the January 1989 issue of the journal.

Drs. Robbins and Wolff were invited participants in the Fogarty Scholar Symposium on Control of the Thyroid Gland, March 1989, organized by Prof. Ragnar Ekholm and Dr. Seymour Wollman (NCI). Dr. Robbins delivered the opening address entitled, "The Thyroid as a Model Endocrine System", and Dr. Wolff gave a paper on "Excess Iodine Inhibits the Thyroid by Multiple Mechanisms".

Dr. Robbins delivered the major lecture at the Workshop on Thyroid Cancer at Johns Hopkins University School of Medicine, April 1989, on the subject of "The Therapeutic Use of I-131". Dr. Robbins also organized a Clinical Center Staff Conference on "Thyroid Cancer: A Lethal Endocrine Neoplasm" on March 28, 1990, with participants from the NCI Departments of Surgery, Pathology and Radiation Epidemiology, the Clinical Center Department of Nuclear Medicine and his own group in CEB.

The 4th Edelhoch Memorial Lecture was delivered by Alvin Taurog, Professor of Pharmacology, University of Texas, Southwestern Medical School on "The Mechanism of Action of Antithyroid Compounds" on March 9, 1990.

A number of countries were represented by the visiting fellows and scientists in the Branch, including France, Greece, India, Italy and Japan.

## I. Thyroid Biochemistry and Pathophysiology

### A. Thyroid hormone-Protein Interactions

It was previously demonstrated that high density lipoproteins (HDL) are the major lipoprotein carriers of thyroxine (T<sub>4</sub>) in human plasma, and that apoA-I,

the main protein component, has a single T4 site of medium affinity. Using monoclonal antibodies obtained from Dr. L. Curtiss, La Jolla, CA, and the method of photoaffinity labeling with underivatized inner-ring labeled T4, the location of the T4 binding site was shown to reside within the N-terminal third of the molecule. It was also found that the pattern of inhibition of T4-affinity labeling was altered in the intact HDL particle, apparently due to conformational changes induced in apoA-I by interaction with lipids.

Similar studies were performed with human low density lipoproteins (LDL) and their apolipoprotein, apoB-100, using monoclonal antibodies obtained from Dr. Y. Marcel, Montreal, Canada, and from Dr. Curtiss. It was confirmed that apoB-100 contains three binding sites for T4, and these were localized to regions within the N-terminal quarter (residues 474-539) the midportion (residues 1438-1481) and the C-terminal quarter of the molecule. It was also shown that these T4 binding sites are outside the LDL receptor binding domain(s) of apoB-100 and distant from its 13 heparin binding sites.

Future studies are planned to assess the possible physiological role of the T4-lipoprotein interactions, since lipoproteins are internalized by specific cell surface receptors and also may function in the release of lipids from cells. Presumably, thyroid hormone can participate in these interactions (Benvenge, Cahnmann, Robbins).

#### B. Thyroid Hormone Metabolism

Thyroid hormones must cross the plasma membrane of cells to interact with nuclear or other intracellular receptors. In brain cells, most of the triiodothyronine (T3) in the nucleus is derived intracellularly from T4, and it has been demonstrated previously that mouse neuroblastoma cells (NB41A3) internalize T4 by a saturable, stereospecific, energy dependent transport mechanism. In further characterization of this transport, it has been shown that L-system neutral amino acids, in particular L-phenylalanine at physiological concentrations, are competitive inhibitors of both T3 and T4 transport. In the presence of 0.1mM L-phenylalanine, plasma membrane transport of thyroid hormones increased more than 2-fold, whereas 1.0mM L-serine or D-phenylalanine had little effect. This interaction of L-system amino acid and thyroid hormone transport may be of physiological importance.

The binding of thyroid hormones to isolated plasma membranes was studied in NB41A3 neuroblastoma cells. Saturable binding of L-T3 to two classes of sites was observed ( $K_a$   $8.4 \times 10^9$  and  $7.3 \times 10^6 M^{-1}$ ). Affinity labeling with bromoacetylated thyroid hormone disclosed stereospecific binding to SDS-PAGE bands with approximate molecular masses of 27 kDa (preferentially labeled by BrAc-L-T3), 32 kDa (by BrAc-D-T3) and 48 and 87 kDa (by BrAc-L-T4). The 27 kDa protein accounted for 3.4% of total BrAc-L-T3 binding and may be involved in intracellular transport of L-T3.

3,5-Dibromo-3'-pyridazinone-L-thyronine (L-94901), a member of a novel class of thyromimetics, reduces hepatic cholesterol synthesis with little effect on cardiac function in rats. Because receptor binding of L-94901 in isolated heart and liver nuclei are similar but in vivo binding was 50-fold greater in liver nuclei, studies on the drug's effect on plasma membrane transport of T3 was studied. Kinetic analysis of the initial rate of uptake in three cell lines showed noncompetitive inhibition, with  $K_i$  for liver and brain cells 10-fold lower than for muscle derived cells. This effect on plasma membrane

transport may explain the differential effect in the intact animal and is further evidence for the pathophysiological importance of active plasma membrane transport of thyroid hormone into cells (Lakshmanan, Goncalves, Cahnmann, Robbins).

### C. Thyroid Hormone Action

The availability of thyroid hormone receptor protein in large amounts should facilitate elucidation of thyroid hormone action at the molecular level. To accomplish this, the baculovirus-Sf9 insect cell system was used to overexpress the rat alpha thyroid hormone receptor (TR $\alpha$ ) and its nonligand-binding variant form (TR $\alpha$ VI). The coding region of the viral polyhedrin gene was replaced with the rTR $\alpha$  or rTR $\alpha$ VI genes by recombination in Sf9 cells, and the recombinant virus was purified by plaque assay using T3 binding activity. This hormone binding activity in recombinant virus infected cells was found only in nuclei and accounted for about 15% of the nuclear protein. A phosphorylated 48 kDa protein in nuclear extracts showed specific binding to the thyroid hormone response element (TRE) of the rat malic enzyme gene. In the case of RV-rTR $\alpha$ VI infected cells, a unique 58 kDa protein band was observed. Thus, Sf9 cells were able to overexpress the T3 receptor related genes and can be a source for receptor purification.

At least two complexes of P32-labeled TR $\alpha$  and malic enzyme TRE were demonstrated by gel shift assay and footprint analysis. This suggested that several receptor molecules can bind to the TRE either as monomers or multimers. As expected, the Sf9-expressed rTR $\alpha$ VI did not bind to the malic enzyme TRE but instead it inhibited the formation of one of the rTR $\alpha$ -TRE complexes. This suggests that there are two rTR $\alpha$  species in Sf9 cells and that only one of them can form a hetero- or homodimer (Mitsuhashi, Hallenbeck, Nikodem).

The previously demonstrated regulatory proteins that bind to the promoter region of the malic enzyme gene have been isolated and cloned. One of these designated MPE2 (malic promoter element 2) contains a 10 bp direct repeat located 70 bp upstream from the cap site. The potential importance of this region is suggested by its presence in several other genes including mouse and human histocompatibility genes, c-fos gene promoter, and several viruses that are pathogenic in humans (such as HIV and cytomegalovirus). Thus the transcriptional protein binding to this element might be involved in regulating a number of different genes involved in human disease.

An attempt to purify these proteins from rat liver nuclei by chromatographic procedures is in progress. In addition, a direct cloning technique has been employed that has succeeded in isolating a 1.6 kb rat cDNA clone for a 35 kDa protein. This protein does not contain the common DNA binding motifs such as zinc fingers, homeo boxes, leucine zippers or POU domains, but is rich in proline and glutamine residues, a feature of other transcription factors. The nucleotide sequence of the rat DNA is >95% homologous with that of the human, and it hybridizes to a 1.6 kb mRNA in most rat and frog tissues, at all developmental stages in Xenopus, and in several cell lines. Thus this evolutionarily conserved gene may be essential for cell survival. Studies are in progress to assess the role of this putative transcriptional factor in gene expression and to determine whether it has a role in thyroid hormone responsiveness (Petty, Raptis, Nikodem).

In previous work, the thyroid hormone response element (TRE) of the malic enzyme (ME) gene was identified as occupying the region -281/-261 in the 5' flanking region. To examine whether the ME TRE can function independently, a synthetic double stranded oligonucleotide containing the ME TRE sequence -287/-257 was incorporated into the linker region of the pBLCAT2 reporter plasmid. In this vector, CAT expression is driven by the thymidine kinase promoter and is not altered by the presence of cotransfected T3 receptor and T3 treatment. Introduction of a single copy of ME TRE conferred a very high T3 inducibility ( $\approx 500$  fold) in the presence of rTR $\alpha$  and T3. Two copies increased T3 responsiveness further by a factor of 2. Part of this high degree of inducibility by T3 was shown to be due to a marked inhibitory effect of rTR $\alpha$  in the absence of hormone. Thus, the ME TRE sequence could not only function independently of the ME gene as an enhancer conferring T3 responsiveness to a heterologous promoter, but it also could act as a repressor in the presence of overexpressed rTR $\alpha$  (Desvergne, Nikodem).

The ME TRE contains two interesting features: an imperfect direct repeat of the T(T/A)GGGG(T/A) sequence (-281/-266), flanked on either side by an AGGAC(G/A) sequence. A study of synthetic mutants showed that either 3' or 5' deletion of the latter sequence resulted in substantial loss of T3 responsiveness, indicating that both were required for maximal T3 inducibility of the thymidine kinase promoter. On the other hand, mutations in the former sequence showed unequal effects on T3 responsiveness when guanine residues at opposite ends were modified.

The mutated TREs were also used to assess their DNA binding properties by a gel shift competition assay. The degree of competition for TR $\alpha$  binding to the ME gene was directly proportional to the level of T3 response for a given DNA sequence. This demonstrates that binding of TR $\alpha$  to the TRE is required for its function (Petty, Mitsushashi, Desvergne, Nikodem).

The critical period of brain development is marked by the onset of active myelination of nerve fibers and this process is greatly reduced by neonatal hypothyroidism. Myelin basic protein (MBP) comprises 30-40% of myelin in the central nervous system, and the MBP gene is one of the set of neural differentiation specific genes required for brain maturation. In this study, the modulation of MBP gene expression by thyroid hormone was investigated in mice and rats during and after development. These species are convenient to study because much of brain development occurs postnatally.

Total MBP mRNA was measured by Northern blot hybridization using a MBP cDNA coding for the full size 18.5 kDa isoform of MBP, and isoforms ranging from 14 to 21.5 kDa were analyzed by RNase protection assay. It was found that thyroid hormone was not required for the initiation of MBP gene expression in mice since all four isoforms were detected on the 4th postnatal day. Hypothyroidism, however, resulted in a 3 to 4 fold decrease in the amount of total RNA. Among the isoforms, the greatest decrease (9- and 17-fold, respectively) was seen in mRNA encoding 21.5 and 18.5 kDa MBP on the 18th postnatal day, when these isoforms are maximally expressed. At other times and for other isoforms the decrease ranged from 2- to 4-fold.

MBP mRNA in the brain of adult mice and rats rendered hypothyroid at 5 weeks of age and killed 4 weeks later was decreased 3- to 4-fold, indicating unexpectedly that thyroid hormone still affects MBP gene expression in young adults (at about the time of sexual maturity). In rats thyroidectomized at 10 weeks, however, hypothyroidism had no effect. Thyroxine treatment reversed the effect of hypothyroidism in young adults, but excess thyroxine administration for 12 days did not alter MBP mRNA levels.

Studies are in progress to determine the mechanism of the thyroid hormone action, and preliminary data from run-on transcription assay in 13 week old rats showed no effect on the rate of transcription of the MBP gene. Further experiments with younger animals are required before it can be decided whether the hormone acts on transcription or posttranscriptionally. Both stages of gene expression are regulated by thyroid hormone in other systems (Farsetti, Nikodem, Mitsuhashi, Robbins).

#### D. Studies in Thyroid Disease

The most effective therapy for differentiated thyroid cancer that has spread after surgical removal of the primary tumor is internal radiation with iodine-131. However, ablation of the cancer is often incomplete and the beneficial effects must be balanced against undesired exposure of radiosensitive normal tissues. Two forms of adjuvant therapy designed to improve the effectiveness of I-131 are under study. The first is lithium ion, which has the unique property of slowing the release of organic iodine from thyroid follicles without interfering with iodide uptake. Preliminary findings in this ongoing study were previously reported, showing that lithium can slow the usually rapid turnover of iodine in some cases of thyroid cancer.

The second form of adjuvant therapy is the addition of a radiosensitizing drug. Patients with high risk thyroid cancer are being recruited into a randomized study to compare conventional I-131 therapy with I-131 combined with low-dose doxorubicin (adriamycin, 10 mg/m<sup>2</sup>). Preliminary data indicate that the combined therapy does not introduce untoward effects from the therapy, but it is too early to evaluate any possible benefit. It may be necessary to involve other institutions in this study in order to enlist the required number of high-risk cancers patients.

In addition to whole body scanning with I-131, the serum thyroglobulin concentration provides an accurate marker for the presence of differentiated thyroid cancer after the thyroid gland has been removed. Since some patients with positive thyroglobulin assay have negative whole body diagnostic scans, the effect of a therapeutic dose of I-131 in such patients is under study. All of 11 patients had detectable I-131 accumulation in the post-therapy scans. On subsequent study, 2 patients reverted to a status of negative thyroglobulin. In 3 patients with persistently positive thyroglobulin, additional I-131 therapy did not reveal I-131 uptake on post-therapy scans. Long-term follow-up is required to evaluate the possible benefit of such therapy (Ain, Shiver, Robbins).



## II. Mechanisms of Cell Secretion

Studies have continued on microtubules, a component of the cytoskeleton that is involved in cell secretion. Previously developed methods were employed to elucidate further the structural modifications in tubulin and their effect on polymerization and other properties. Using the polarity-sensitive dye, Nile red, and time resolved spectral analysis it was shown that the nature of the buffer composition affected the molecular conformation. In glutamate buffer, in contrast to Mes buffer, polymerization resulted in a red shift, reduction in fluorescence intensity and a decrease in lifetime, suggesting an increase in polarity of the binding environment.

The thermodynamics of tubulin dimer polymerization was evaluated by short-column equilibrium sedimentation. This newly perfected technique permits complete analysis within 2 min, thus avoiding tubulin degradation during the run. Temperature dependence of polymerization from 2°-30°C gave a linear van't Hoff plot and positive values for the enthalpy and entropy changes on association:  $\Delta S^\circ = 38.1 \pm 2.4 \text{ cal deg}^{-1} \text{ mol}^{-1}$ , and  $\Delta H^\circ = 2.1 \pm 0.7 \text{ kcal mol}^{-1}$ , and a small or zero value for the heat capacity change,  $\Delta C_p^\circ$ , on association. The entropically driven association of tubulin monomers was analyzed in terms of the importance of hydrophobic interactions to the stability of the monomer association, and compared to the thermodynamics of dimer polymerization.

The effect of small conformational changes on state changes of tubulin was studied in order to understand the progressive loss during tubulin "aging" of its ability to polymerize and to bind colchicine and ANS. This denaturation was evaluated by examining the effects of urea. Taxol-driven polymerization of tubulin was inhibited about 50% by 0.3M urea abolished by 1M urea. Binding of colchicine analog was increased over this low range of urea concentration. Fluorescence analysis suggested that the urea effects were the result of changes in the environment of the binding site, associated with a "tightening" of the protein. The lack of accessibility into or out of the binding site was supported by the finding that accessibility to proteolysis was also reduced in urea. At higher urea concentrations (3 to 5M) the molecule becomes "loosened" and eventually unfolds (Wolff, Sackett, Bhattacharyya, Zimmerman).

## III. Adenylate Cyclase of Bacterial Origin

The virulence factor of Bordetella Pertussis, previously shown to be an invasive, extrabacterial adenylate cyclase, has been purified by a rapid two-step chromatographic procedure. Previous purification methods gave a homogeneous catalytically active cyclase without invasive properties. By protecting the invasive property in urea, the fully active virulence factor has now been obtained in good yield. It is a single polypeptide possessing highly potent enzymatic activity (0.4 mmol/mg/min) and invasive activity (0.5  $\mu\text{mol}$ /mg enzyme/mg cell protein/min) and retains calmodulin sensitivity. Purification also removed a separate invasion inhibitor. The virulence factor has a molecular mass of 175 kDa, and partial sequence analysis indicates that it is a product of the recently cloned cya genes. Thus the virulence factor is a single molecule with two domains: a relatively stable catalytic domain in the amino terminal portion, and a labile domain required for penetration into host cells (Wolff, Gentile, Sackett, Raptis).

### III. Mechanisms of Embryogenesis

During amphibian embryogenesis, realization of the body plan is dependent on the formation of two important axes, the dorsal-ventral and the anterior-posterior. The latter, established during gastrulation, requires two major events: the acquisition of polarity by the dorsal mesoderm followed by the induction of ectoderm. To understand the molecular mechanism of these events, a gene that may be involved, XPO, has been characterized. The XPO gene was cloned from a subtraction library consisting of *Xenopus* genes differentially expressed during gastrulation. The XPO cDNA clone (4.2 kb) was sequenced and is unique except for an 18 amino acid sequence that is >75% homologous with the zinc finger contained within GAG, the small nucleic acid binding protein of retroviruses. The N-terminal region of the deduced protein contains a heptad repeat which is not a classical "leucine zipper". The mRNA is present at early gastrulation, reaches a peak during neurulation, and disappears by the late tailbud stage. By stage 11 the transcript is localized mainly to posterior mesoderm and ectoderm, and at stage 13 it is found in the dorsal/posterior region of the embryo, restricted to mesoderm and ectoderm. A small amount is found in the anterior ectoderm adjacent to the neural plate.

Synthetic mRNA encoding the complete open reading frame of XPO, or a frame shift mutant RNA, was injected into embryos at the 2-cell stage. A delay in gastrulation occurred in half of the injected embryos and led to a diminished anterior axis. This suggests that overexpression or misexpression of XPO may disturb the formation of the anterior-posterior axis.

It has been suggested that retinoic acid regulates anterior-posterior formation in *Xenopus* embryos. To understand the role of XPO in this process, ectoderm induction assays were conducted with retinoic acid and other growth factors and the expression of XPO and other markers of anterior-posterior development was assessed. Preliminary data indicate that treatment of blastula stage embryos with XTC factor plus retinoic acid results in greatly increased XPO expression. Three other markers are decreased. This is the first direct evidence that retinoic acid can redirect the type of mesoderm in an embryo (Sato, Sargent-NICHD).

Transcription factors containing the POU domain sequence (found in the genes *Pit-1*, *Oct-1* and 2, and *unc-86*) may act as regulators of tissue-specific gene expression during embryogenesis, but little is known about the early specification of pituitary and neural tissue. To study this, four different POU domain genes were cloned from *Xenopus laevis* using a PCR strategy. The cDNA derived from adult pituitary and brain poly A+ mRNA served as a template. Sequence analysis of one pituitary-derived clone (XPOU-2) showed 96% homology with the rat *Pit-1* gene within the POU domain region. XPOU-2 is a single copy gene and its transcript was found only in pituitary. XPOU-1, -3 and -4 were derived from brain, and all were expressed in early *Xenopus* embryos as well as in brain. XPOU-1 also appears to be a single copy gene and contains a POU domain most closely related to the rat genes *Brain 1*, *Brain 2* and *Testes 1*. Its transcripts (2.5 and 3.0 kb) accumulated in as early as stage 14 embryos (neural plate stage) and expression continued throughout the tadpole stage. At stage 22-23 (tailbud stage), XPOU-1 transcripts were localized in the head and dorsal portions of the embryo. They were also present in adult brain and skin, but not in testes, liver, heart, muscle or kidney. Antipeptide antibodies

directed against the deduced amino acid sequence of XPOU-1 are in preparation. These will be used to assess the expression of XPOU-1 protein and its localization in the embryo during development. They will also be tested for their ability to perturb normal development (Sato, Agarwal).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroxine-Protein Interactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. Robbins	Chief CEB, NIDDK
Others:	S. Benvenga	Guest Researcher CEB, NIDDK
	H.J. Cahnmann	Scientist Emeritus CEB, NIDDK

## COOPERATING UNITS (if any)

Dr. R.E. Gregg, (NHLBI)

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Hormone Metabolism and Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We previously demonstrated that high density lipoproteins (HDL) bind T4 in human plasma through a single binding site on apoA-I and that low density lipoproteins (LDL) bind T4 through 3 binding sites on apoB-100. By photoaffinity labeling with [<sup>125</sup>I]T4 and its inhibition by monoclonal antibodies to the apolipoproteins, we have investigated the location of the T4 binding sites. In apoA-I, the T4 site is in the N-terminal third of the molecule. In apoB-100, the T4 sites are outside the LDL receptor binding domain and are located in the molecular domains designated as apoB-26, apoB-44 and apoB-30. Conformational changes due to interaction with lipids appear to result in altered patterns of monoclonal antibody inhibition.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45004-19 CEB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure of Polypeptide and Protein Hormones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: K. Prasad

Visiting Associate, CEB, NIDDK

Others: R. E. Lippoldt

Health Services Ofcr., CEB, NIDDK

## COOPERATING UNITS (if any)

Washington University School of Medicine, St. Louis, MO (Dr. J.Heuser); Temple University School of Medicine, Philadelphia, PA (Dr. J.H.Keen)

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Protein Structure Section

## INSTITUTE AND LOCATION

## TOTAL MAN-YEARS:

NIDDK, NIH, Bethesda, Maryland 20892

PROFESSIONAL

OTHER

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been discontinued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45009-23 CEB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies in Thyroid Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: J. Robbins Chief, Clinical Endocrinology Branch CEB, NIDDK  
Others: K. Ain Medical Staff Fellow CEB, NIDDK  
T. Shiver Medical Staff Fellow, CEB, NIDDK  
M. Phyllaier Technician, CEB, NIDDK

COOPERATING UNITS (if any)

Dr. J.Norton, Surgery Branch, NCI; Dr.J.Reynolds, Nuclear Medicine, CC; Dr. M. Merino, NCI; Dr. C. Meyers, NCI

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL

1.7

OTHER:

.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two forms of adjuvant therapy designed to improve the effectiveness of I-131 radiation in thyroid cancer are under study. The first is lithium ion, which is used to prolong the retention of I-131 in the cancer. Preliminary findings have been described in previous annual reports. The second is the use of doxorubicin (adriamycin) as a radiosensitizing agent. To evaluate its effectiveness, we have begun a randomized study of high-risk thyroid cancer patients who receive standard I-131 therapy with or without the administration of low-dose adriamycin (10mg/m<sup>2</sup>) 30 min preceding each I-131 treatment dose. To our knowledge, this is the first attempt to randomize patients in a therapy protocol for thyroid cancer employing I-131.

Other patients with negative I-131 whole body scans but elevated concentration of thyroglobulin in blood are given I-131 therapy in an attempt to localize the cancer by post-therapy scanning. Most patients have been found to have positive post-therapy scans and they are under evaluation for possible beneficial effects of the therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45014-19 CEB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membranes and Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Wolff Associate Chief CEB, NIDDK

Others: D. L. Sackett Senior Staff Fellow CEB, NIDDK  
T. M. Shiver Medical Staff Fellow, CEB, NIDDK  
L. Knippling Technician CEB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.75

PROFESSIONAL:

1.5

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In pursuing our interest in the role of the cytoskeleton in adrenal cell function, we have been treating Y-1 cells with acrylamide and evaluating rounding, steroid, and c-AMP production. Treatment with acrylamide results in non-cAMP mediated steroid production in the Y-1 cell with morphological changes similar to those induced by forskolin. Acrylamide acts after a 2 1/2 hour latent period at a step prior to pregnenolone formation. Steroid production is similar to that produced by colchicine, slightly less than that produced by ACTH stimulation, and considerably less than steroid production by forskolin; these results imply that, if rounding of cells represents cytoskeletal rearrangement, it is non cAMP mediated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45016-20 CEB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroid Hormone Secretion and the Function of Microtubules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Wolff Associate Chief CEB, NIDDK

Others: D.L. Sackett Senior Staff Fellow CEB, NIDDK  
B. Bhattacharyya Visiting Scientist (10 mos) CEB, NIDDK  
D. Zimmerman Guest Worker (2 mos) CEB, NIDDK  
T. Shiver Medical Staff Fellow CEB, NIDDK

COOPERATING UNITS (if any)

Jay Knutsen, NHLBI

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.05

PROFESSIONAL

1.8

OTHER:

0.25

CHECK APPROPRIATE BOXES:

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The domain structure of the  $\alpha$  and  $\beta$  tubulin monomers and their association to the noncovalent dimer has been investigated by several independent approaches: binding of the polarity sensitive dye nile red, and equilibrium centrifugation with a newly developed short column method. Using steady state and time resolved fluorescence we find that binding of nile red to tubulin enhances and blue shifts fluorescence emission to about 623 nm with a "shoulder" around 665 nm. Binding is reversible and saturable, has lifetimes of 4.5 ns and 0.6 ns fluorescence in Mes buffer associated with the 623 nm peak and the 665 nm shoulder, and has rotational correlation times of >50 ns for the 4.5 ns lifetime and 0.3 ns for the 0.6 ns lifetime. Dilution of tubulin in Mes results in an apparent red shift of emission without lifetime changes, due only to loss of the 623 nm component. The more "nonpolar" site is located in a region of subunit-subunit contact which accounts for the fluorescence changes upon dilution and permits estimation of a subunit dissociation constant of  $1\mu\text{M}$ .

Because of the lability of tubulin, a rapid, short-column equilibrium centrifugation method was developed in which equilibrium is attained in a few hours. Monomer-dimer distribution analysis yields a dissociation constant of  $2 \times 10^{-7}\text{M}$  at  $5^\circ\text{C}$ . Thermodynamic analysis show that dimerization is entropically driven.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45018-15 CEB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenylate Cyclase and Other Extracellular Products of B. Pertussis

PRINCIPAL INVESTIGATOR (Last other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. Wolff	Associate Chief CEB, NIDDK
Others:	L. Knipling	Technician CEB, NIDDK
	F. Gentile	Visiting Fellow (4 months)
	D.L. Sackett	Senior Staff Fellow

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL

OTHER:

1.5

0.75

0.75

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A rapid two-step purification to homogeneity of the calmodulin-activated adenylate cyclase from urea extracts of Bordella pertussis organisms (strain 114) is described. Catalytic and invasive activities are purified 30- and 177-fold, respectively, and virtually no degraded forms are found. Specific activities are 0.4 mmol/min/mg and 0.5  $\mu$ mol/mg enzyme protein/mg cell protein/min for catalytic and invasive activities, respectively. The 15 amino terminal amino acids agree with those deduced from the DNA sequence, as does the molecular mass of 175 kDa (guanidine) or 177 kDa (urea) obtained by equilibrium sedimentation. The larger apparent molecular mass seen in SDS PAGE can be ascribed to anomalous migration. Half maximal cyclase activation occurs at  $3-4 \times 10^{-10}$  M calmodulin in the presence of  $\text{Ca}^{2+}$  and at  $2 \times 10^{-8}$  M calmodulin in its absence.  $\text{Ca}^{2+}$  activation is maximal at 60-100  $\mu$ M free  $\text{CaCl}_2$  (at low calmodulin concentrations), and free  $\text{Ca}^{2+}$  concentrations above  $\approx 125$   $\mu$ M are inhibitory at any calmodulin concentration. Extracellular  $\text{Ca}^{2+}$  is essential for intoxication. In CHO cells exogenous CaM does not inhibit penetration of the cyclase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 45028-12 CEB
PERIOD COVERED <b>October 1, 1989 to September 30, 1990</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders ) <b>Thyroid Hormone-Cell Interactions</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator ) (Name, title, laboratory, and institute affiliation)		
PI:	J. Robbins	Chief CEB, NIDDK
Others:	M. C. Lakshmanan E. Goncalves M. Phyllaiaer H. Cahnmann	Medical Staff Fellow CEB, NIDDK Visiting Fellow, CEB, NIDDK Technician, CEB, NIDDK Scientist Emeritus, CEB, NIDDK
COOPERATING UNITS (if any)  None		
LAB/BRANCH	Clinical Endocrinology Branch	
SECTION	Hormone Metabolism and Action Section	
INSTITUTE AND LOCATION <div style="text-align: right;">NIDDK, NIH, Bethesda, Maryland 20892</div>		
TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
2.2	1.8	.4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided )  <p>Thyroid hormones must cross the plasma membrane to interact with nuclear or other intracellular receptors. While a saturable transport system for triiodo-thyronine (T3) has been demonstrated in many types of cells, this is less well established for thyroxine (T4). In mouse neuroblastoma cells, we found that T4 was actively and stereospecifically transported. Furthermore, L-system neutral amino acids were shown to be competitive inhibitors of both T3 and T4 transport. The inhibitory effect of phenylalanine may be of physiological importance.</p> <p>The binding of thyroid hormones to isolated neuroblast plasma membranes was studied by affinity labeling with bromoacetyl T3 and T4. There was selective binding of bromoacetyl T3 to a 27 kDa protein component, which may be involved in intracellular transport.</p> <p>3,5-Dibromo-3'-pyridazinone-L-thyronine (L-94901), a novel thyromimetic drug, reduces hepatic cholesterol synthesis with little effect on cardiac function in rats. Kinetic analysis of T3 uptake in myoblasts, hepatoma cells and neuroblasts showed that L-94901 was a noncompetitive inhibitor of T3 uptake in all cell types, but Ki for liver and brain cells was 10-fold lower than for muscle derived cells. This differential effect on plasma membrane transport may explain the observations in the intact animal.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Mapping of Triiodothyronine Responsive Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. M. Nikodem Senior Scientist CEB, NIDDK

Others: K. Petty Research Fellow CEB, NIDDK  
Beatrice Desvergne Visiting Fellow CEB, NIDDK  
A. Raptis Visiting Associate CEB, NIDDK  
M. Phyllaier Technician CEB, NIDDK  
R. Lippoldt Chemist CEB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Hormone Metabolism and Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.7

## PROFESSIONAL:

3.4

## OTHER:

1.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been using the rat hepatic malic enzyme gene as a model to study the mechanism of thyroid hormone action. Herein we have shown that the ME TRE sequence at position -287/-257 of the ME gene 5' flanking region can bind T3 receptor and function as a very potent T3 responsive element either as a repressor of the TK promoter activity (-T3) or an enhancer (+T3). Mutation and deletion analyses of ME TRE revealed that this region contains several important subregions, two of them, separated by 10bp, are essential for the altered transcriptional activity of the TK promoter.

We have also been studying the interactions of the thyroid hormone receptor with other transcriptional factors participating in the assembly of transcription initiation complex. Using ME promoter sequence we have identified a transcriptional factor whose binding activity to the region -50 to -70 is altered by thyroid hormone and thus might determine together with the receptor, the frequency of initiation of mRNA synthesis. To study this interaction the relevant transcriptional factor has to be purified. Using liver nuclear extracts fractionated on a DEAE-sepharose column and heparin-agarose we have partly purified two different proteins that bind to the ME sequence -50/-70. Further purification will be accomplished using a DNA affinity column to generate material for microsequencing and antisera production.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45034-07 CEB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Specific Rat Liver mRNAs by Thyroid Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. M. Nikodem, Ph.D.

CEB, NIDDK

Others: R. Lippoldt

CEB, NIDDK

J.E. Rall

CEB, NIDDK

M-K. Song

CEB, NIDDK

D. Grieco

CEB, NIDDK

COOPERATING UNITS (if any)

Dr. S.M. Aloj and Dr. L.Kohn, LBM, NIDDK

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been kept in abeyance this year pending the results of other projects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
201 DK 45038-03 CEB

PERIOD COVERED  
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Molecular biology of thyroid hormone receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	V.M. Nikodem	Visiting Scientist	CEB, NIDDK
Others:	T. Mitsuhashi	Visiting Fellow	CEB, NIDDK
	P. Hallenbeck	NRC Fellow	CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH  
Clinical Endocrinology Branch

SECTION  
Hormone Metabolism and Action Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.9	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

In *spodoptera frugiperda* (Sf9) cells, both rTR $\alpha$  and rTR $\alpha$ vI were localized in nuclei, were partly extractable by salt (0.4 M NaCl) and were phosphorylated. In band shift assay, Sf9-expressed rTR $\alpha$  formed two complexes with malic enzyme TRE, and both were readily recognized by the receptor specific antibody. Footprint analysis of these complexes showed an asymmetric binding of rTR $\alpha$  to malic TRE which indicates that thyroid hormone receptor binds as a monomer and dimer to its target sequence. Co-expression of rTR $\alpha$  and rTR $\alpha$ vI resulted in the decrease of only the upper complex. This observation suggests that there are two rTR $\alpha$  species in Sf9 cells, only one of which can form homodimer or heterodimer.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of thyroid hormone on synthesis of myelin basic proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Farsetti

Visiting Fellow, CEB, NIDDK

Others: V. Nikodem  
T. Mitsuhashi  
J. RobbinsSenior Investigator, CEB, NIDDK  
Visiting Associate, CEB, NIDDK  
Chief, CEB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Hormone Metabolism and Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

1.8

## PROFESSIONAL

1.8

## OTHER

## CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Thyroid hormone is not required for the initiation of brain myelin basic protein (MBP) gene expression which occurs on the 4th postnatal day in mice. MBP mRNA encoding all four major MBP isoforms are detected on that day in the absence of thyroid hormone. Hypothyroidism results in a 3- to 4-fold decrease in total MBP mRNA concentration in newborn mice. Among the isoforms, all of which are decreased by hypothyroidism, the greatest decrease (9- and 17-fold, respectively) is seen in mRNA encoding 21.5 and 18.5 kDa MBP on the 18th postnatal day, when these isoforms are maximally expressed. At other times and for other isoforms the decrease ranges from 2- to 4-fold. MBP mRNA in the brain of adult mice and rats rendered hypothyroid at 5 weeks of age and killed 4 weeks later is decreased 3 to 4 fold. This indicates that hypothyroidism still affects MBP gene expression in young adults (at about the time of sexual maturity). In rats thyroidectomized at 10 weeks of age, hypothyroidism has no effect. Thyroxine treatment reverses the effect of hypothyroidism in young adults, but excess thyroxine administration for 12 days does not alter MBP mRNA levels. The manuscript describing this study has been prepared for publication.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The role of POU-domain genes during Xenopus laevis embryogenesis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Sato Senior Staff Fellow, CEB, NIDDK

Others: V. Agarwal Visiting Fellow, CEB, NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Section on Developmental and Molecular Endocrinology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transcription factors containing the POU domain sequence (found in the genes Pit-1, Oct-1 and 2, and unc-86) may act as regulators of tissue-specific gene expression during embryonic development. Little is known about the early specification of pituitary and neural tissue. To study these developmental processes, we have cloned at least four different POU domain genes from Xenopus laevis. Degenerate oligonucleotides representing all possible codons for two conserved 7 amino acid regions (IKLGYTQ and WFCNRRQ) flanking the POU domain were used as primers in the polymerase chain reaction (PCR). The cDNA derived from adult pituitary and brain poly A+ mRNA served as a template for the PCR. Sequence analysis of one clone derived from pituitary cDNA (XPOU-2) showed 96% homology with the rat Pit 1 gene within the POU-domain region. XPOU-2 is a single copy gene and its transcript (~4.5 KB) is found only in pituitary. We have sequenced at least 3 different POU-containing cDNA's from brain (XPOU-1, 3, and 4). All of these genes are expressed in early Xenopus embryos as well as in brain. The XPOU-1 transcripts (2.5 KB, 3.0 KB) appear to be localized in the head/dorsal region of a tailbud embryo. The smaller transcript of XPOU-1 is also present in adult skin. A partial cDNA sequence of XPOU-1 shows no homology outside the POU-domain. Antipeptide antibodies directed against the deduced amino acid sequence of XPOU-1 are currently being pursued and should be useful for examining the expression of the XPOU-1 protein.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45042-01 CEB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of XPO, a Localized Zinc Finger Gene, in Anterior-Posterior Axis Formation in *Xenopus*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Sato Senior Staff Fellow, CEB, NIDDK

## COOPERATING UNITS (if any)

Dr. Tom D. Sargent, Laboratory of Molecular Genetics, NICHD

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Section on Developmental and Molecular Endocrinology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The anterior-posterior axis, established during gastrulation, requires 2 major events: first, the acquisition of polarity by dorsal mesoderm and second, the induction of ectoderm by the dorsal mesoderm. We are interested in understanding the molecular basis of the formation of the anterior-posterior axis and have characterized a gene, XPO (*Xenopus*-posterior), which may be involved in this process. The XPO cDNA clone (4.2 KB) has been sequenced and is completely unique except for an 18 amino acid sequence that is >75% homologous to the zinc finger contained within GAG, the small nucleic acid binding protein of retroviruses. Additionally, the N-terminal portion of the deduced protein contains a heptad repeat which is not a classical "leucine zipper". The mRNA is present in early gastrulating embryos, reaches its peak during neurulation, and disappears by the late tailbud stage. At stage 11 the transcript appears to be highly localized in posterior mesoderm and ectoderm with a small component present in the anterior ectoderm. At stage 13, the transcript was also observed in the anterior ectoderm in a region adjacent to the neural plate. A small amount of transcript was also observed in the anterior ectoderm in a region adjacent to the neural plate. In an animal ectoderm induction assay, XPO has been shown to be induced by both basic FGF and XTC factor.



ANNUAL REPORT OF THE DIABETES BRANCH  
National Institute of Diabetes and Digestive and Kidney Diseases

Investigators in the Diabetes Branch conduct clinical investigation and basic scientific research with special emphasis on understanding the mechanism of action of insulin and the causes of diabetes mellitus. Towards this end, a multi-disciplinary approach has been applied, involving techniques of molecular biology, cell biology, biochemistry, and clinical physiology. Other projects include studies of hormones and other messenger molecules important to the regulation of growth and development, especially growth hormone and insulin-like growth factors I and II. In addition, there is an active research program with respect to acromegaly, a clinical disease caused by the overproduction of growth hormone. Finally, there is research into the evolutionary origins of the endocrine system, and also into the possible role of hormones and peptide growth factors in the mammalian central nervous system.

Recognition of previous achievements

The contributions of investigators in the Diabetes Branch have been recognized in many ways. First, several investigators have received research support from private funding agencies: the Juvenile Diabetes Foundation, the American Diabetes Association, and the Diabetes Research and Education Foundation. Second, two Section Chiefs in the Diabetes Branch, Dr. Phillip Gordon and Dr. Jesse Roth, serve as Director and Scientific Director respectively of the NIDDK. Third, investigators in the Diabetes Branch serve on the Editorial Board of several internationally respected journals. Fourth, the senior members of the Diabetes Branch receive frequent invitations to lecture at national and international meetings. Fifth, over the years several individuals in the Branch have been recognized by receiving awards for their research. This year Dr. Simeon Taylor received the Distinguished Service Award from the USPHS.

The insulin gene and its regulation of expression

Insulin is a hormone synthesized predominantly in the  $\beta$ -cell of the pancreas. In addition, investigators in the Diabetes Branch have presented evidence that the hormone is synthesized in other locations as well. To pursue investigations of the regulation of expression of the insulin gene in both the pancreas and elsewhere, recent studies have employed the species *Xenopus laevis*, an important experimental model system to study early development. Two non-allelic genes for insulin have been identified in this species, and the cDNAs encoding these two insulins have been cloned. A new technique involving the polymerase chain reaction has been developed to amplify insulin cDNA. This new technique has been used to detect minute quantities of insulin mRNA even in unfertilized *Xenopus* oocytes. The insulin mRNA is of maternal origin, and

disappears shortly after fertilization of the egg. During embryonic development, transcription of the insulin gene begins again shortly after neurulation. At this time, insulin mRNA localizes primarily in the most cephalic regions. This raises the possibility that insulin may play a role in the development of the central nervous system. Further evidence that insulin may be synthesized in extrapancreatic tissues has been obtained in studies carried out in the rat. The polymerase chain reaction has been used to amplify insulin cDNA synthesized from mRNA of tissues including brain, liver and testis. Based on these studies, it is possible to detect levels of insulin mRNA in these tissues that are estimated to be approximately 0.001 to 0.01% of that detected in the pancreas.

Insulin is the hormone that is the most important to regulation of glucose metabolism. Defects in insulin secretion can cause diabetes. For the most part, at the time individuals are diabetic, their insulin levels tend to be low. However, in patients destined to develop noninsulin-dependent diabetes, hyperinsulinemia is frequently present prior to the development of diabetes. To evaluate whether hyperinsulinemia may play a role in causing insulin resistance in diabetes, a transgenic mouse model has been developed. In this transgenic mouse, the human insulin gene is overexpressed in the  $\beta$ -cell. Thus, these mice are hyperinsulinemic because of the simultaneous expression of both murine and human insulin. The degree of hyperinsulinemia in these transgenic mice correlates with the number of copies of the human insulin gene integrated into the genomic DNA of the mice. These mice develop mild insulin resistance and also hyperglycemia, suggesting that primary hyperinsulinemia can lead to glucose intolerance and insulin resistance, both abnormalities typical of noninsulin-dependent diabetes.

Other studies have been carried out directly in human subjects. Two patients have been identified who have mutations in their insulin gene. Both of these patients are heterozygous for a mutation substituting histidine for arginine at position 65 in the proinsulin molecule. This mutation impairs the proteolytic processing of proinsulin to insulin. The possibility that this type of mutation in the insulin gene may play a role in the development of the common form of noninsulin-dependent diabetes mellitus has been evaluated by determining the nucleotide sequence of the insulin gene in several patients with noninsulin-dependent diabetes mellitus, including Pima Indians and Nauruans, two ethnic groups with a high prevalence of diabetes. In all cases, the sequence of the insulin gene appears to be normal. Therefore, no evidence could be found that mutations in the insulin gene contributed to the pathogenesis of diabetes in these patients.

### Insulin receptors

The insulin receptor is a heterotetrameric glycoprotein located on the cell surface. It is encoded by a single gene

that encodes a precursor molecule. The precursor undergoes multiple post-translational processing steps including proteolytic cleavage to yield two different types of subunits: an  $\alpha$ -subunit that contains the insulin binding site and a  $\beta$ -subunit that is a tyrosine specific protein kinase. When insulin binds to the extracellular domain of the receptor, this activates the tyrosine kinase associated with the intracellular domain of the receptor. Activation of the tyrosine kinase plays a necessary role in mediating insulin action. Several projects in the Diabetes Branch pertain to various aspects of insulin receptor biosynthesis and insulin receptor function.

To study the regulation of the expression of the insulin receptor gene, the DNA in the 5' flanking region of the human insulin receptor gene has been cloned. Deletion analysis has been employed to identify a 70-base pair region of the gene that contains the majority of the promoter activity. In addition, a weak enhancer has been identified upstream of the promoter. Further investigations are directed towards identifying other regulatory domains that may play a role in the developmental and endocrine regulation of the expression of the insulin receptor gene.

In another project, several steps involved in post-translational processing of the insulin receptor during biosynthesis are being investigated. In particular, N-linked glycosylation, O-linked glycosylation, and fatty acylation are being studied. The role of N-linked glycosylation is being investigated by site-directed mutagenesis to eliminate the N-linked glycosylation sites. By expression of the cDNAs encoding these mutant receptors, it may be possible to determine the functional role of the N-linked glycosylation. Progress has also been made toward identifying the O-linked glycosylation site in the  $\beta$  subunit.

The role of the tyrosine kinase in mediating insulin action is also being investigated. A 120 kilodalton glycoprotein in rat liver membranes has been identified to serve as a substrate for phosphorylation by the insulin receptor. This glycoprotein has been immunoaffinity purified, a partial amino acid sequence has been determined, and a cDNA encoding this glycoprotein has been obtained. This protein substrate, pp120, appears to be identical to a previously identified enzyme that has the property of hydrolyzing ATP and GTP. The effect of phosphorylation to regulate the enzymatic activity of this protein is presently being investigated. By expressing the cDNA through transfection, it should be possible to determine the physiological role of this protein, and what role the protein may have in mediating the effect of insulin upon target cells.

In addition, as part of a long term collaboration with scientists at the University of Geneva, studies are underway to explore the mechanism and significance of receptor-mediated endocytosis, receptor internalization, and recycling.

Considerable progress has been made in identifying mutations in the insulin receptor gene, and elucidating the role of this type of genetic defect in causing human disease. Multiple different mutations have been identified in the insulin receptor gene, these fall into five classes: class 1, mutations that inhibit insulin receptor biosynthesis, frequently by decreasing levels of insulin receptor mRNA; class 2, mutations that impair the transport of mutant receptors to the cell surface; class 3, mutations that decrease the affinity with which insulin is bound to the receptor; class 4, mutations that impair tyrosine kinase activity; class 5, mutations that accelerate receptor degradation, apparently by inhibiting recycling of internalized receptors back to the plasma membrane. Most of these mutations have been identified in patients with relatively rare genetic syndromes associated with severe insulin resistance and acanthosis nigricans. At present, studies are underway to determine the prevalence of this type of mutation, and also to investigate the possibility that this type of mutation may contribute to the pathogenesis of more common diseases such as noninsulin-dependent diabetes mellitus and polycystic ovary disease.

A novel technique utilizing positron emission tomography (PET) has been employed to study insulin receptors in vivo in rhesus monkeys. A method has been developed to rapidly synthesize a high specific activity  $^{18}\text{F}$  derivative of insulin appropriate for utilization in PET scanning. Studies in monkeys have demonstrated that the  $^{18}\text{F}$  insulin derivative binds to insulin receptors in vivo. The binding is rapid, specific, and reversible. In the case of the liver, the binding becomes irreversible approximately 5 minutes after injection, possibly as a result of internalization of the peptide into the hepatocyte. In the case of the kidney, a significant component of the  $^{18}\text{F}$  insulin binding remains displaceable for a longer period of time. Excretion of the  $^{18}\text{F}$  is predominantly in bile and urine.

#### Insulin-like growth factors and insulin-like growth factor receptors

Previously, cDNAs encoding IGF-I have been cloned from rat liver. In addition, part of the rat IGF-I gene has been cloned. More recently, two non-allelic IGF-I genes have been cloned from the species *Xenopus laevis*. Regulation of the expression of the rat IGF-I gene has been studied extensively. This gene, subsequent to transcription, undergoes a complex pattern of splicing. There are at least three 5'- untranslated region sequences, the steady-state levels of which are differentially regulated in vivo by growth hormone in a tissue-specific manner. In addition, there are two alternative splicing patterns of the 3' end that result in two different propeptides being encoded. These two alternative E-domains of the propeptides are the result of inclusion or exclusion of exon IV. The two different E-domains differ with respect to the

presence or absence of two potential N-linked glycosylation sites. Studies using an in vitro rabbit reticulocyte translation system together with canine microsomes have demonstrated that these potential N-linked glycosylation sites actually undergo glycosylation, at least in vitro. The physiological significance of these alternative splicing patterns is presently being investigated.

During post-natal life, the principal recognized role of IGF-I is to promote growth. The possibility that IGF-I has a role in regulating embryonic development is being investigated using the chicken embryo system. IGF-I can be detected in the yolk of the fertilized chicken egg. Moreover, the chicken IGF-I gene is expressed in the blastoderm and the level of expression increases during organogenesis. The regulation of the expression of the IGF-I gene differs in different tissues. For example, in brain and pancreas, IGF-I is expressed during mid-embryogenesis while in the liver its expression is only significant after hatching. The mechanism whereby IGF-I regulates differentiation and development is being examined using the lens of the eye. In the lens, IGF-I stimulates the expression of the gene encoding  $\delta$ -crystallin. At present, the cis-acting elements and trans-acting factors that mediate the regulation of  $\delta$ -crystallin by IGF-I are being mapped. The effects of IGF-I to regulate transcription have also been studied in another system. In cultured neuronal and glial cells, IGF-I, insulin, and phorbol esters all increased the expression of a gene encoding the rat brain/HepG2 glucose transporter (GLUT 1).

The biological effects of IGF-I are mediated through a cell surface receptor, a heterotetrameric glycoprotein that is homologous to the insulin receptor. A cDNA encoding a portion of the IGF-I receptor has been cloned from rat granulosa cells. In addition, partial length cDNAs encoding receptors in this family have been cloned from *Xenopus* embryos. A portion of the 5' flanking region of the IGF-I receptor gene has been cloned in the rat. The promoter region of the gene has been identified. This region contains a unique transcription initiator sequence with a single start site, previously found in a number of developmentally regulated genes. The promoter region lacks a TATA box, but contains putative Sp1 sites.

#### Growth hormone and acromegaly

A long term follow-up study of patients with acromegaly has been conducted in the Diabetes Branch over the past 25 years. Several modes of therapy have been employed, including radiation therapy, trans-sphenoidal hypophysectomy, and more recently treatment with drugs such as bromocriptine and somatostatin analogs. Recent studies have demonstrated that somatostatin analogs can be extremely useful in lowering growth hormone levels in patients with acromegaly. In addition, this agent can be useful in reducing hormonal hypersecretion caused

by tumors of the pituitary that secrete thyroid stimulating hormones (TSH) and also in glucagon secreting tumors of the pancreas. Unfortunately, somatostatin analogs have a side effect of causing the patients to develop thickened bile. Because of the previous observation that patients with somatostatin secreting tumors have a high prevalence of gallstones, it seemed important to determine how frequently treatment with somatostatin analogs would also cause this complication. At present, a study is underway to evaluate this possibility.

### Evolution of the endocrine system

Previous work from the Diabetes Branch has provided evidence suggesting the synthesis of vertebrate-type peptide hormones in unicellular organisms as well as other primitive organisms that had not been recognized to synthesize these hormones. Recently, considerable progress has been made in identifying and characterizing one such peptide in great detail. An ACTH-like peptide has been purified from *E. coli*. The amino acid sequence of this ACTH-like molecule was identical to the C-terminal 33 amino acids of elongation factor II, a protein that is required for protein synthesis. This ACTH-like molecule had a sequence that is similar to vertebrate ACTH. In addition, synthetic peptides with the same sequence as the *E. coli* ACTH-like molecule bind to anti-ACTH antibodies. Furthermore, these synthetic peptides possess ACTH-like bioactivity in a bioassay using a vertebrate system. These results suggest that vertebrate ACTH-like molecules may have arisen evolutionarily from more primitive, but related molecules - in this case elongation factor II.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47001 09DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Phosphorylation of the Insulin and IGF-I Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	D. LeRoith	Section Chief,	DB/NIDDK
Others	C.T. Roberts, Jr.	Research Biologist	DB/NIDDK
	Z. Shen-Orr	Guest Researcher	DB/NIDDK
	H. Werner	Visiting Fellow	DB/NIDDK
	M. Adamo	Staff Fellow	DB/NIDDK
	B. Stannard	Biologist	DB/NIDDK
	M. Woloschak	Research Associate	DB/NIDDK

## COOPERATING UNITS (if any)

University of Florida (M. Raizada)

## LAB/BRANCH

Diabetes Branch

## SECTION

Section of Molecular and Cellular Physiology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

## Summary

Insulin and insulin-like growth factor (IGF-I) receptors in the brain are similar in structure to their non-neural peripheral counterparts. However, they have smaller apparent molecular weights and differ in the glycosylation of their subunits. In addition, the role of insulin and IGF-I in neuronal cells differ when compared to other cells. To investigate the mechanisms for these differences we have begun studies that include structure and function studies of the insulin and IGF-I receptors in brain. To this end we have cloned and sequenced the rat IGF-I receptor cDNA and its proximal promoter. These tools will allow us to study regulation of gene expression of this receptor. In addition, since early metabolic actions of these hormones include glucose uptake, we have also investigated the gene expression of the brain-type glucose transporter (glut 1) in neuronal and glial cells in culture.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1,-1989 to September 20, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Gene Expression and Insulin Action

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	F. De Pablo	Visiting Scientist			DB/NIDDK
	J. Roth	Director, DIR			DB/NIDDK
Others:	J. Serrano	Visiting Associate	L. Scavo	Guest Worker	DB/NIDDK
	A. Shuldiner	Senior Staff Fellow	L. Scott	IRTA	DB/NIDDK
	L. Marban	Guest Worker	K. Tanner	Spec. Vol.	DB/NIDDK
	J. Alemany	Visiting Fellow			DB/NIDDK
	R. Dashner	Microbiologist			DB/NIDDK

## COOPERATING UNITS (If any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Receptor and Hormone Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.5

## PROFESSIONAL:

4.5

## OTHER:

3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Molecular biological techniques are being used to discern gene expression of insulin, insulin-like growth factors (IGFs) and their receptors and actions in several vertebrate model systems. Insulin is apparently a requirement for normal development, and the chicken embryo is one of the suitable models for studying the role of insulin in development. The ontogeny of expression of the insulin gene has been studied and the gene for IGF-I and IGF-I receptor are cloned. The role of insulin in cell differentiation and gene expression in the eye lens of chick embryo is also being used as a cell model to understand the action of insulin in development. The amphibian, Xenopus laevis, is also a model system used to study development. The amphibian insulin and insulin-like growth factor I genes have been isolated and their sequences determined and confirmed by molecular cloning. Studies are in progress to define the expression of these peptides during Xenopus development using the polymerase chain reaction.

A transgenic mouse line with multiple copies of the human insulin gene integrated into its genome has been established. The degree of hyperinsulinemia correlates with human gene copy numbers. The transgenic mice provide a model system for studies in regulation of insulin gene expression and the effects of chronic hyperinsulinemia on glucose homeostasis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47005 - 18 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of insulin receptors in circulating cells in man

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director	NIDDK
Others:	S.I. Taylor	Chief	DB/NIDDK
	C. Hendricks	Biol. Lab Tech.	DB/NIDDK
	R. Arakaki	Senior Staff Fellow	DB, NIDDK
	O. Rodriguez	Special Volunteer	DB, NIDDK

## COOPERATING UNITS (if any)

Wayne State University (G. Grunberger)

## LAB/BRANCH

Diabetes Branch

SECTION Clinical and Cellular Biology Section; Biochemistry and Molecular Pathology  
Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The present work continues prior investigations of insulin receptors on circulating cells in patients with insulin resistance and diabetes mellitus. The effects of diet, fasting and treatment on receptor function are under investigation. Insulin receptors are evaluated for their ability to bind insulin and to act as tyrosine-specific protein kinases.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47007 1<sup>5</sup> DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antibodies to Receptors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.

S. Taylor

Chief,

DB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Biochemistry &amp; Molecular Pathogenesis Section

## INSTITUTE AND LOCATION

NIDDK/NIH/Bethesda, Md.

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (a1) Minors☐ (a2) Interviews☒ (b) Human tissues☐ (c) Neither

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I N A C T I V E

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47009 - 03 DB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Positron Emission Tomography

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J. Roth	Director, DIR,	NIDDK
Others:	R. Eastman	Clinical Director, DB/NIDDK	
	M. Lesniak	Chemist	DB/NIDDK

COOPERATING UNITS (if any)

Ervin Baas, NIDDK; Kenneth Jacobson, NIDDK; Michael Channing, Bonnie Dunn, Richard Carson, Nuclear Medicine; John Biebe, VDM.

LAB/BRANCH

Diabetes Branch

SECTION

Receptor and Hormone Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A substituted  $^{18}\text{F}$  insulin derivative has been synthesized for study of insulin receptors in vivo by positron emission tomography (PET). Using a novel prosthetic group methodology  $^{18}\text{F}$  insulin can be prepared in 4 hours with a specific activity of 4-12 curies/micromole at the time of administration. In vivo studies have been performed in 7 Rhesus monkeys to determine whether the  $^{18}\text{F}$  insulin binds to insulin receptors in vivo. Under basal conditions there is rapid, specific, and reversible uptake of the label by liver and kidney at tracer concentrations in the low picomolar range. Binding to liver is irreversible 5 minutes after injection, while a significant component of kidney uptake can be displaced, suggesting different kinetics for insulin uptake in these tissues. Excretion of the radioactivity is predominantly in bile and urine.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47014-21 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Acromegaly and Growth Hormone

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director	NIDDK
Others:	C. M. Hendricks	Bio. Lab. Tech.	DB, NIDDK
	R. F. Arakaki	Senior Staff Fellow	DB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Clinical and Cellular Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL

1.0

## OTHER:

.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Acromegalic patients have continued to be followed with respect to pituitary irradiation. Further, we are evaluating the effects of transsphenoidal hypophysectomy in these patients and comparing them to the pituitary-irradiated patients.

A group of patients in a long-term follow-up study was evaluated to determine the effect of joint disease as a function of time following pituitary radiation. It appears that the joint disease is a function of the age of the patient and/or the degree of involvement at initial therapy. Thus in patients with relatively severe joint disease, the joint disease progresses in spite of very significant reductions in growth hormone following radiation treatment. Other studies underway are attempting to determine the possible effect of radiation on pituitary function or other complications of therapeutic maneuver.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47018 13DB

## PERIOD COVERED

October 1, 1989 September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Cellular Hormone-Like Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J. Roth	Director (DIR)	DB/NIDDK
	D. LeRoith	Section Chief	DB/NIDDK
Others	A. Liotta	Expert	DB/NIDDK
	C.T. Roberts, Jr.	Research Biologist	DB/NIDDK
	L. Sankaran	Chemist	DB/NIDDK
	M. Adamo	Staff Fellow	DB/NIDDK
	M. Bach	Med. Staff Fellow	DB/NIDDK
	H. Foyt	Med. Staff Fellow	DB/NIDDK

## COOPERATING UNITS (if any)

1. Laboratory of Cellular Developmental Biology, NIDDK (J. Shiloach)
2. Univ. Maryland, Dept. Obstetrics and Gynecology (E. Adashi)

## LAB/BRANCH

Diabetes Branch

## SECTION

Section of Molecular &amp; Cellular Physiology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Summary

Insulin, Adrenocorticotrophic hormone (ACTH), somatostatin, and other vertebrate peptide hormones have been demonstrated in unicellular organisms e.g. E. coli, and higher plants e.g., spinach. Using protein purification techniques including high performance liquid chromatography (HPLC) and affinity chromatography molecules similar to ACTH have been purified to homogeneity. The sequence of these molecules suggest that it may be part of the C-terminal 33 amino acids of elongation factor II of E. coli and may represent the evolutionary origins of vertebrate ACTH. Molecular biology techniques will be used to establish this concept. In addition, rat IGF-I cDNAs have been cloned to assist in the screening for insulin-related genes in primitive organisms. They have also been used to study gene expression in hepatic and non-hepatic rat and human tissues.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47019 - 13 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphologic studies of ligand binding to cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.

P. Gorden

Director

NIDDK

## COOPERATING UNITS (if any)

Institute of Histology and Embryology,  
University of Geneva School of Medicine, Geneva, Switzerland.  
(J.L. Carpentier, L. Orci) - Foreign

## LAB/BRANCH

Diabetes Branch

## SECTION

Clinical and Cellular Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This work represents over 12 years of collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of receptor-mediated endocytosis similar to other biologically important ligands that bind to cells. In the present study, using electron microscopy, we find there is anatomical correlation between the dissociation of  $^{125}\text{I}$ -insulin and its localization on the cell surface. This work has now been extended to include an insulin-resistant cell line that has an abnormal surface which leads to a higher association of ligand to the non-villous portion of the cell surface. Further, receptor-mediated endocytosis also appears to be regulated in hypoinsulinemic states. In both rat and in human type I diabetes there is an inhibition of  $^{125}\text{I}$ -insulin internalization in the hyperglycemic state: the normal state is restored by insulin treatment. The role of intracellular calcium on the endocytotic process, as well as the relationship of stimulators of protein kinase C to internalization of both insulin and unrelated ligands such as transferrin, have been studied also. In addition, the function of the small non-coated invaginations in receptor-mediated endocytosis is being investigated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47022 - 11 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin receptors in syndromes of extreme insulin resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Simeon Taylor	Section Chief,		
Others:	Alessandro Cama	Visiting Associate	Phillip Gorden	Director
	Takashi Kadowaki	Visiting Fellow	Jesse Roth	Director, DIR
	Hiroko Kadowaki	Special Volunteer	Eiichi Imano	Vis. Fellow.
	Domenico Accili	Special Volunteer	R. L-Toledano	Vis. Fellow
	Catherine Mckeen	Expert	Lucy Sierra	Biologist
	Richard Arakaki	Sen. Staff Fellow	F. Barbetti	Vis. Fellow
	Carla Hendricks	Biotech.	Nina Raben	Vis. Assoc.

COOPERATING UNITS (if Matthew M. Recheler, MCNEB, NIDDK)

Luitgard Mosthaf, Axel Ullrich - Max Planck Institute, Munich, Germany; Manuel Serrano-Rios - Centro Especial Ramon y Cajal, Madrid, Spain; Steve Lillioja - NIH - Phoenix Medical Center; John Merenich - Fitzsimmons Army Medical Center; Kenneth H. Gabbay - Baylor Medical School, Houston, TX

LAB/BRANCH

Diabetes branch

SECTION

Biochemistry &amp; Molecular Pathogenesis

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

9.5

PROFESSIONAL:

7.5

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin resistance contributes to the pathogenesis of several disease states including obesity and noninsulin-dependent diabetes mellitus. We have investigated the insulin receptor gene in patients with genetic forms of insulin resistance to gain insight into biochemical defects that give rise to disease.

Five classes of mutations have been identified:

1. Impaired receptor biosynthesis, due to either decreased levels of insulin receptor mRNA and/or premature chain termination mutations.
2. Impaired transport of receptors to the plasma membrane, due to missense mutations in the extracellular domain of the receptor.
3. Decreased affinity of insulin binding.
4. Decreased activity of the insulin receptor tyrosine kinase.
5. Accelerated receptor degradation associated with resistance to the effect of acid pH to dissociate insulin from its receptor within the endosome.

Studies are presently underway to estimate the prevalence of mutations in the insulin receptor gene in order to determine whether mutations in this gene contribute to the pathogenesis of the common form of noninsulin-dependent diabetes mellitus.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 47024 - 11 DB
PERIOD COVERED October 1, 1989 to September 30, 1990		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Biosynthetic Labeling of the Insulin Receptor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	E. Collier	Expert DB/NIDDK
Others:	H. Caro R. Sayers R. Arakaki P. Gorden	Visiting Fellow DB/NIDDK Biologist DB/NIDDK Senior Staff Fel. DB/NIDDK Director NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH                      Diabetes Branch		
SECTION                          Clinical and Cellular Biology Section		
INSTITUTE AND LOCATION      NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)  <p>           We have studied the post translational modifications of the <u>insulin receptor</u>, i.e., <u>glycosylation</u>, <u>fatty acylation</u>, and phosphorylation using biosynthesis labeling of the insulin receptor. After labeling the insulin receptor with radioactive sugars, the individual subunits were isolated and tryptic peptides made from each subunit. These peptides were separated using high performance liquid chromatography and the glycosylated peptides detected by counting the fractions in a liquid scintillation counter. Based on the differential sensitivity of these labeled peptides to peptide: N-glycosidase F, an enzyme that removes high mannose and complex N-linked oligosaccharides, and endo-a-N-acetylgalactosaminidase, an enzyme that removes O-linked oligosaccharides, we have shown that the insulin receptor contains <u>O-linked oligosaccharides</u>. These O-linked carbohydrates are on the beta subunit. The peptide containing the O-linked oligosaccharides was localized to the amino terminus of the beta subunit. An anti-peptide antibody made to the 12 amino acids at the amino terminus of the beta subunit was able to immunoprecipitate specifically the tryptic fragment containing the N- and O-linked oligosaccharides. Using an inhibitor of O-linked glycosylation, we investigated the function of the O-linked glycosylation. In the presence of this inhibitor the binding of insulin to the insulin receptor was unchanged.         </p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47025-07 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tissue Receptors for Insulin and Insulin-like Growth Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J. Roth	Director, DIR	DB/NIDDK
Others:	M.A. Lesniak	Chemist	DB/NIDDK
	M. Rojeski	Senior Staff Fellow	DB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Receptor and Hormone Action Section

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previously we had reported the presence of insulin in extracts of rat brain and other extrapancreatic tissue. Although the synthesis of extrapancreatic insulin is unknown, its concentration in some tissue, e.g. brain, testes, is independent of insulin concentration in blood. Presently, we have detected insulin mRNA in liver, testes and brain subsections using reverse transcriptase and polymerase chain reaction (RT-PCR). In in situ hybridization histochemistry on brain tissue slices using an antisense oligonucleotide probe there was an accumulation of granules in discrete brain regions. These data suggest that insulin in brain and other extrapancreatic tissues may be of local origin.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 47026 - 06 DB
PERIOD COVERED October 1, 1989 to September 30, 1990		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Tyrosine-specific protein kinase activity associated with the insulin receptor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	Simeon Taylor	Chief DB, NIDDK
Others:	Domenico Accili Sonia Najjar	Visiting Scientist DB, NIDDK IRTA DB, NIDDK
COOPERATING UNITS (if any) University of Catanzaro, Catanzaro, Italy (Nicola Perrotti) Howard University, Washington, DC (Ronald Margolis) Johns Hopkins University, Baltimore, MD (Ann Hubbard)		
LAB/BRANCH Diabetes Branch		
SECTION Biochemistry & Molecular Pathogenesis		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		

In the first step in insulin action, insulin binds to its receptor on the surface of the target cell. The insulin receptor is a transmembrane protein that possesses tyrosine-specific protein kinase activity. When insulin binds to the extracellular domain of the receptor, this activates the receptor tyrosine kinase activity. A growing body of evidence suggests that the activation of the receptor's tyrosine kinase is a necessary step in initiating the biological actions of insulin. Accordingly, we have embarked upon a search for intracellular proteins that are substrates for phosphorylation by the receptor-associated tyrosine kinase. We have identified one such substrate in rat liver plasma membranes: a glycoprotein with an apparent molecular weight of 120,000 (pp 120). In addition to being a substrate for the insulin receptor, pp120 can be phosphorylated by the receptors for epidermal growth factor and insulin-like growth factor I. pp 120 is present in liver from several species, but has not been identified in other tissues. The glycoprotein (pp120) was immunoaffinity-purified using monoclonal antibody HA4. Based on partial amino acid sequence data, pp120 has been tentatively identified as ectoATPase - an enzyme associated with hepatocyte plasma membranes. Studies are underway to express ectoATPase by transfection of cDNA in tissue culture cell lines. This will facilitate answering the question of whether tyrosine phosphorylation regulates the enzymatic activity of ectoATPase.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 47027-05 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of SMS 201-995 in Hormone Secreting Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director		NIDDK
Others:	R. F. Arakaki	Senior Staff Fellow	DB,	NIDDK
	A. Shuldiner	Senior Staff Fellow	DB,	NIDDK

## COOPERATING UNITS (if any)

B. Weintraub Chief, MCNEB, NIDDK

## LAB/BRANCH

Diabetes Branch

## SECTION

Clinical and Cellular Biology Section

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have studied the use of the long-acting somatostatin analogue, SMS 201-995, in patients with acromegaly, TSH secreting pituitary tumors, glucagonomas and insulinomas. These studies have defined 1) an appropriate dose and schedule for control of TSH secreting pituitary adenoma and its resultant hyperthyroidism; 2) an appropriate subgroup of acromegalic patients in whom this analogue, given thrice daily, controls GH hypersecretion; and 3) the effects of the drug in glucagonoma syndrome in terms of control of glucagon hypersecretion and correction of hypoaminoacidemia. Our current studies have focused on the long term use of this agent in acromegaly and patients with TSH secreting tumors and the correlation of hormonal effects with symptomatic benefit. In addition, our studies indicate that all patients develop thickened bile accumulation in the gallbladder while receiving treatment, which may progress to gallstones.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47028-01 DB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of the Insulin Receptor Gene

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Catherine McKeon	Expert	DB, NIDDK
Assoc. Inv.	Simeon Taylor	Chief	DB, NIDDK
Others:	Tony Pham	Spec. Vol.	DB, NIDDK
	Gillian Walker	Spec. Vol.	DB, NIDDK
	Domenico Accili	Vis. Assoc.	DB, NIDDK
	Takashi Kadowaki	Fogarty Fel.	DB, NIDDK

## COOPERATING UNITS (if any)

Paola Salvatore, University of Naples, Italy

## LAB/BRANCH

Diabetes Branch

## SECTION

Biochemistry &amp; Molecular Pathophysiology

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

2.5

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Regulation of the number of insulin receptors on the cell surface plays a critical role in determining insulin sensitivity. Previously, we have demonstrated that the insulin receptor is regulated at the transcriptional level by glucocorticoids. In order to study the mechanism of transcriptional regulation of the insulin receptor gene, we have cloned the 5' end of the human insulin receptor gene. We have begun to characterize the promoter and find it has many features of a "housekeeping gene". The 5' flanking region of the gene has been sequenced and is extremely G-C rich. Using primer extension, multiple transcription starts sites have been identified. Using an expression vector, we have localized the promoter activity to a 70 bp region by deletion analysis. We have localized a weak enhancer upstream of the promoter. In addition, this promoter demonstrates the unusual property of functioning equally well in either orientation. Further studies to identify sequences responsible for the transcriptional regulation are underway.

ANNUAL REPORT OF THE CLINICAL HEMATOLOGY BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

Study of Immunology of Blood Cell Deficiencies

**Objectives:** To study the immunochemistry of immune disorders affecting blood cells and effects of the immune reactions on cellular physiology, biochemistry and in vivo cellular kinetics.

**Methods employed:** Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens; microanalyses for nitrogen, histamine, and alkaloid drugs; quantitative measurements of complement fixation, cellular agglutination and precipitin reactions; immunoelectrophoresis; methods of provoking antibody responses in man and animals; and isotopic and fluorescent labelling applied to antigens and antibodies. Tissue culture techniques; red cell agglutination techniques; lymphocyte transformation tests; autoradiography; electron microscopy; and high pressure liquid chromatography for analyses of drugs and synthesized analogs. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling. Methods of cell membrane component analysis involving cellulose transfer techniques, immunoprecipitation, isoelectric focussing, electroelution, microtiter enzyme immunoassays, and anion exchange chromatography. Monocyte separation techniques; cellular arachidonate metabolism, protein and in RNA synthesis using labeled precursors; specific mRNA assays for IL6, IL1, and TNF; intracellular Ca<sup>++</sup> flux measurements.

**Major findings:**

1. A Unique Immunoglobulin-binding Protein from Platelets

**Findings:**

We previously identified an intracellular platelet protein of 95 kD that binds all classes of immunoglobulins present in most normal sera. We have further characterized the protein and studies its variation in disease in attempts to correlate its unusual attributes with a physiologic function. Patients with the thrombocytopenic disorders ITP, drug purpura, post transfusion purpura and thrombocytopenia of systemic lupus erythematosus and HIV infection were not found to have significant variations in the 95 kD proteins. Additional characteristics of the protein that we defined are a pI of 5.65, no glycosylation, apparent association with a 47 kD fragment of gelsolin, high percentages of aspartic and glutamic acids and alanine and leucine, blocked N-terminal amino groups, similarity to protein we identified in HEL cells.

**Planned work:**

- Generate internal sequence data from proteolytic digests for comparison with known proteins and potential cloning utilizing an HEL cell cDNA library.
- Study immunoglobulin transport in platelets to determine if the 95 kD protein functions as a chaperone protein for transfer of plasma Ig's to alpha granules.

2. Posttransfusion Purpura (PTP), a Manifestation of Intercellular Transfer of Plasma Membrane Proteins

**Findings:**

PTP is a disease caused by alloantibodies against transfused platelet antigens. Sensitized patients develop thrombocytopenia 5 to 8 days post-transfusion. Pathophysiology of this occurrence has been obscure, hypotheses ranging from

autoantibodies, to cross-reactive antibodies to antigen-antibody AgAb complexes. We found that normal plasma in blood bank blood contains 1 to 3% of platelet membrane, alloantigenic components in a nonsedimentable form associated with integrin molecules that adsorb to fresh platelets added to ultracentrifuged plasma. This occurs equally well in the presence of excess antibody against specific platelet alloantigens. Patients with PTP were found to have circulating nonsedimentable foreign platelet antigen during thrombocytopenia persisting as long as 4 weeks after transfusion. The antigen in PTP plasma was adsorbable onto normal platelets in vitro. These findings show that adsorption of AgAb complexes involving transfused platelet alloantigen is the basis for platelet destruction in sensitized individuals. Quantitation of reactions indicates that as little as 2-4% of the antigenic content of 1 unit of blood can initiate and perpetuate PTP and that as few as 200-400 molecules of AgAb complex per platelet is sufficient to cause thrombocytopenia.

#### Planned work:

- a. To further characterized nonsedimentable platelet membrane material that can be exchanged between cells.
- b. To determine significance of integrin moieties of nonsedimentable platelet membrane material in the pathophysiology of other thrombocytopenic disorders such as neonatal purpura and ITP and its potential involvement in cytoadhesion events leading to thrombosis or arteriosclerosis.

### 3. Experimental Therapy of Immune Neonatal Thrombocytopenia

#### Findings:

We continue to participate as one of 6 laboratories in an international study of prophylactic therapy for immune neonatal thrombocytopenia. Of 34 cases referred to us by local physicians, 12 were found to be caused by alloimmune sensitization of  $Pl^{Al}$  antigen by serologic studies in our laboratory, 1 to possible HLA sensitization, and the others to non immunologic cause, such as eclampsia, fetal asphyxia, or sepsis. The national combined results on over 200 cases indicate that adrenocorticosteroids that cross the placenta and I.V. IgG given to the mother for a 2 week antepartum period prevent fetal hemorrhage at birth when alloimmunity is the cause of neonatal thrombocytopenia. A final report on the study is forthcoming.

### 4. Cellular Immunity in Thrombocytopenia States

#### Findings:

The potential role of specific immunocyte responses in the pathophysiology of antibody-mediated thrombocytopenias has not been investigated. Since antibody (Ab) coupled to monocyte Fc-receptors will initiate monocyte activation steps when antibody binds to target antigen (Ag) we have used a prototypic platelet alloantigen system to "arm" monocytes. Known antibodies against the  $Pl^{Al}$  platelet antigen were coupled to normal elutriated monocytes. The armed monocytes (AM's) were exposed to adherent platelets containing  $Pl^{Al}$  and cultured for varying periods before harvest. AM's aggregated around the platelets whereas control monocytes did not. The AM's exposed to  $Pl^{Al}$  did not increase general protein or mRNA synthesis and showed no significant increases in mRNA for interleukin (IL) 6 or tumor necrosis factor (TNF) but did show a minor increase in IL1 mRNA production. AM's prelabeled with  $^3H$ -arachidonate prior to exposure to  $Pl^{Al}$  produced significant increases in arachidonic acid (AA) by 16 hours and an increase in calcium flux was noted with  $^{45}Ca$ -prelabelled AM's within the first hour of exposure to  $Pl^{Al}$ .

#### Planned work:

- a. Further experiments are planned to confirm the above findings.
- b. Immunologic specificity of relationships between calcium and arachidonate metabolism need corroboration and identification of specific AA metabolites will be pursued.

#### Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

**Objective:** To study reactions and interactions of coagulation factors in vitro and in vivo, to define further the nature of the blood coagulation mechanism, and to study the physiology and biochemistry of platelet responses to agonists causing exocytosis and aggregation. These studies are aimed at determining factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis and at developing better forms of therapy for these diseases.

**Methods Employed:** Methods of protein purification and characterization, techniques of enzymology, proteolytic enzymes and their inhibitors, kinetic analysis of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactive substrates, particularly arachidonate metabolism and phosphorylation. Tissue culture techniques, pharmacologic and physiologic studies of cellular secretion, and platelet aggregation. SDS-PAGE molecular weights, amino acid composition of purified proteins, immunologic RIA, fluorescent, Western Blot, ELISA and other quantitative and qualitative methods of identifying specific epitopes with antibodies, HPLC, affinity columns, immunoprecipitation, and isoelectric focusing.

#### Major findings:

1. Metabolic aspects of platelet activation by crosslinking agents.

#### Findings:

The anion channel blocking drug, diisothiocyanato stilbene sulfonic acid (DIDS), activates platelets to secrete and aggregate by crosslinking amines of platelet plasma membrane structures. Our initial experiments suggested that a span of 12 Å (a single DIDS molecule) is necessary for optimal crosslinking and activation. Current work indicates that spontaneously formed dimers, trimers or larger units of the DIDS molecule in solution, also may induce activation. We are conducting a structure-activity relationship study of DIDS configurations in collaboration with Dr. Brian DeCosta of the Medicinal Chemistry Branch, NIDDK.

We have investigated whether DIDS and thrombin share the same second messengers and activation pathways. Thrombin activation is thought to proceed through G-proteins and phospholipase C to release inositol phosphates and diacylglycerol. We found that DIDS elevates inositol phosphates in platelets to the same extent as thrombin utilizing platelets prelabelled with 3H-inositol. In p32-labelled platelets, DIDS caused phosphorylation of a 47 kDa protein which is known to be the substrate for protein kinase C (PKC). Since PKC is activated in the presence of increased levels of diacyl glycerol, the activation of this enzyme and the release of inositol phosphates suggest that phospholipase C is involved in DIDS-induced platelet activation.

The DIDS-induced cell surface crosslinking followed by activation is similar to certain growth factors such as epidermal growth factor, which activate cells by dimerizing their receptors to turn on the receptor's intrinsic tyrosine kinase. These kinases self-phosphorylate and also have been reported to tyrosine

phosphorylate phospholipase C, possibly inducing its activity. We investigated whether tyrosine phosphorylation has any role in thrombin- and DIDS- induced platelet activation. Thrombin produces tyrosine phosphorylation (TP) of at least seven distinct proteins with molecular weights of 30, 40, 74, 80, 120, 130 and 200 kDa.

#### Planned work:

a. To characterize DIDS receptor(s) by a variety of techniques used in our laboratory. (See Methods Employed.)

b. To determine activation responses of other cell types to DIDS, particularly granulocytes and lymphocytes, and compare characteristics of membrane receptors and signal-transfer biochemistry with those of platelets.

#### 2. Mechanism of thrombin-induced tyrosine kinase activation in platelets

Thrombin-induced TP correlates well with the onset and extent of platelet serotonin secretion suggesting that the two may be related. Direct second messenger elevation with calcium ionophore or with synthetic diacylglycerol has been reported not to induce platelet TP and the role of G-proteins in the process is controversial. We found that direct activation of G-proteins in whole cells by NaF (in the presence of 10  $\mu$ M aluminum fluoride) produced TP similar to that induced by thrombin. NaF at 30-50 mM concentration induced TP of 80 and 130 kDa proteins but TP decreased at 100-200 mM NaF that produced maximum serotonin secretion. We found that low levels of the ionophore A23187 also induced TP of the 80 and 130 kDa but as with NaF, TP decreased when A23187 concentrations were sufficiently high to induce secretion. Thus the TP of the 80 and 130 kDa proteins appears to be effected by minimum elevations of  $Ca^{++}$  that are insufficient to promote secretion.

We used the isoflavone, genistein, a specific tyrosine kinase inhibitor, to investigate the functional role of TP proteins in thrombin-induced platelet secretion. Genistein is reported to be ~20 fold more potent against isolated tyrosine kinases than against serine/threonine kinases. We found that genistein inhibited thrombin-induced platelet secretion and TP with a  $IC_{50}$  of 200 and ~500  $\mu$ M, respectively. It also inhibited thrombin-induced activity of myosin light chain kinase (MLCK) ( $IC_{50}$  200  $\mu$ M) as evidenced by decreased p32 phosphorylation of the 20 kDa myosin light chain. Genistein does not appear to be an effective inhibitor of TP in platelets, suggesting that thrombin tyrosine kinases of activated platelet are relatively resistant to isoflavones. Genistein's inhibitory effect on platelet secretion appears to be mediated by inhibition of MLCK.

a. To isolate and identify the 80 and 130 kDa tyrosine kinase substrates in order to match sequences against possible related conserved sequences of known function or to express them in other cells to identify biochemical changes.

b. To study pathologic conditions involving platelets to determine whether abnormalities in tyrosine kinase activation may be associated with clinical dysfunction.

#### 3. A platelet fibrinogen receptor that modulates secretory responses defined by a unique autoantibody.

Our studies of a patient with a severe bleeding disorder led to discovery of a new autoimmune disease in which the autoantibody inhibits platelet responses to physiologic agonists. The patient's antibody reacted with normal platelets to decrease aggregation elicited by all agonists and to decrease serotonin



secretion by those agonists dependent on platelet reactions with fibrinogen. Platelet metabolic responses preceding to aggregation and secretion were unaffected by the antibody. We found the antibody was directed against the  $Ca^{++}$ -dependent glycoprotein IIb/IIIa complex which it immunoprecipitated, but the antibody does not block reaction of other known allo- or monoclonal anti-GPIIb or -GPIIIa antibodies. The antibody is primarily subclass IgG1 and non-complement fixing. It binds to approximately 80,000 sites/platelet but does not cause platelet destruction or shortened survival.

Planned work:

a. Because this antibody interferes markedly with platelet function but does not affect platelet survival, it has potential value as a therapeutic agent to prevent platelet adhesion and aggregation in treatment of thromboembolic events. We are currently immortalizing the patient's lymphocytes and attempting to select the specific clones for potential pharmacologic antibody production on a CRADA basis.

b. We will attempt to define those characteristics that differentiate antibodies that destroy platelets in vivo at low concentration from antibodies that are compatible with normal platelet survival even present on platelets at high concentrations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51,000-32 CHB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Immunology of Blood Cell Deficiencies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: N. Raphael Shulman

CHB:NIDDK

Others: Diane M. Reid Senior Staff Fellow  
Charles E. Jones ChemistCHB:NIDDK  
CHB:NIDDK

## COOPERATING UNITS (if any)

E. Reed, C. Carter (Blood Bank); J. Bussell, N.Y. Hospital; C. Leisinger (Tulane Univ.)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Decreased platelets resulting from immune reactions is one of the most common causes of clinical hemorrhage. We have studied the following disorders to further understand mechanisms of immune cellular injury and to develop new diagnostic tests and forms of therapy: autoimmune idiopathic thrombocytopenic purpura (ITP); alloimmune and autoimmune neonatal purpura; drug-induced immune purpura; and post-transfusion purpura. The following was accomplished:

We have purified and partially sequenced and are attempting to clone a platelet 95 kD immunoglobulin-binding protein that we recently discovered. This protein is the first eukaryotic cell constituent identified with properties similar to Staph. protein A or Strep. protein G.

Because serologic tests for antibodies responsible for ITP have been uniformly unsuccessful we sought evidence for cellular responses as a basis for pathophysiologic processes and possible diagnostic tests for ITP. Monocytes coupled to known platelet alloantibodies via Fc receptors and exposed to target platelet antigens were found to manifest certain activation steps and/or effector functions. This prototype system is being applied to ITP.

We have further evaluated therapy for alloimmune neonatal thrombocytopenia and established benefits of antenatal therapy with adrenocorticosteroids and I.V. IgG to prevent fetal hemorrhage.

Pathophysiology of posttransfusion purpura has been elucidated by finding that complexes of transfused foreign alloantigen combined with alloantibody adsorb to autologous platelets to cause their destruction. This sheds light on quantitative aspects of intercellular transfer of plasma membrane components and on immune complex disease generally.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51,001-32 CHB

## PERIOD COVERED

October 1, 1939 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Raphael Shulman	Chief	CHB:NIDDK
Others:	Diane M. Reid	Senior Staff Fellow	CHB:NIDDK
	Jaro Vostal	Research Fellow	CHB:NIDDK
	Charles E. Jones	Chemist	CHB:NIDDK

## COOPERATING UNITS (If any)

A. B. Mukherjee (HGB, NICHD) B. Decosta (LMC, NIDDK)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The biochemistry and cellular physiology of platelet secretion has many attributes in common with other secretory cells. Platelet membrane glycoproteins are known to be major factors involved in cell-cell recognition, adhesion, and secretion; but peptide sequences of significance in the various cellular reactions, the biochemistry of signal transmission and the intracellular chain of events leading to activation are only partially clarified.

We have discovered a class of compounds that initiate cellular secretion by cross-linking amine groups of specific plasma membrane proteins of MW ~42,000 and ~66,500 kD plus apparent multimers of 100,000, 140,000 and 200,000 kD. We found crosslinkage activated protein kinase C and phospholipase C. Using a variety of activators and inhibitors to evaluate specific metabolic pathways, we found that crosslinked receptors develop protein kinase activity similar to that elicited by epidermal growth factor and insulin receptors.

To further define metabolic responses of platelets to physiologic agonists we studied mechanisms of thrombin-induced tyrosine kinase activation in platelets and the role of G-proteins in tyrosine phosphorylation (TP). We found TP of 80 and 130 kDa proteins correlated best with G-protein activation and Ca++ elevation but did not appear to be directly linked to secretion.

Further studies of an autoimmune disease we defined last year in which antibody directed at a platelet surface glycoprotein complex interferes with platelet secretion and aggregation, defined a unique epitope that provides new insight into mechanism of agonist-receptor interaction in platelets. Work is underway to clone this antibody for potential therapeutic applications as an anticoagulant.

## ANNUAL REPORT OF THE GENETICS AND BIOCHEMISTRY BRANCH

### NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

#### Biochemical Genetics Section

Dr. Proia and his colleagues have continued their studies of the lysosomal enzyme  $\beta$ -hexosaminidase (a deficiency of which is responsible for Tay-Sachs disease). This enzyme is made up of two subunits  $\alpha$  and  $\beta$ . Over the last few years they have cloned the cDNAs and genes for both chains of the human  $\beta$ -hexosaminidase. In the last year they have isolated near full length cDNAs encoding both subunits of murine  $\beta$ -hexosaminidase. The nucleotide sequence of the  $\alpha$ -subunit has been determined. Genomic clones encompassing portions of the murine  $\alpha$  and  $\beta$ -subunit genes have been isolated. These genes will be used to disrupt the murine  $\beta$ -hexosaminidase genes in embryonic stem cells by gene targeting through homologous recombination.

In a different project Dr. Proia and his colleagues have shown that the Gly<sup>269</sup>->Ser mutation in the  $\beta$ -hexosaminidase  $\alpha$ -subunit can occur in the homozygous form in 3 non-Jewish patients with adult Gm2 gangliosidosis. This is in contrast to the situation in Ashkenazi Jewish patients where the same mutation is always present in compound heterozygosity with a null Tay-Sachs allele.

#### Molecular Genetics Section

Dr. Ackerman and collaborators have continued their work on the mode of action of the *Aspergillus* toxin  $\alpha$ -sarcin and related toxins. They have shown that the toxins  $\alpha$ -sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA. Recently they have shown that microinjection into *Xenopus* oocytes of deoxyoligonucleotides, ribonucleotides and ribozymes

complementary to the same region of 28S RNA abolishes protein synthesis.

On a second project Dr. Ackerman and his colleague have extended their results showing that *Xenopus* oocytes efficiently repair microinjected DNA. They are now investigating which cellular proteins are involved in the repair of UV-induced pyrimidine dimers. They also have succeeded in documenting repair of a chemically modified DNA.

Dr. Hsieh and her colleagues have localized the pairing interactions formed by recombinases to short, defined regions of DNA <60bp in length and have isolated stable structures free of any recombinase protein. These joint molecules have unusual thermal stabilities indicating that they are three-stranded structures. Preliminary experiments indicate that a novel triple-stranded DNA in which the third strand is hydrogen-bonded to an intact duplex DNA may be an intermediate in homologous recombination. Early model building has narrowed down the possibilities for the hydrogen bonding schemes involved. Chemical probing (footprinting) of the stable joints formed is underway to corroborate these interactions.

In a related project, Dr. Camerini-Otero and colleagues have searched for eukaryotic proteins other than recombinases that might either bind to three-stranded DNA or triplex DNA or promote their formation. They have used a gel shift assay to purify and characterize a protein from HeLa cells that binds to the poly(dA)·poly(dT)·poly(dT) triplex. The protein is greatly enriched by passage through a triplex DNA-affinity column.

In another project, Dr. Camerini-Otero and colleagues in order to assess the role that homologous recombination might play in immunoglobulin heavy chain class switching, have sequenced several entire human switch recombination sites, including the S $\mu$  and S $\gamma$ 4 sites. There are significant S $\mu$ -S $\gamma$ 4 matches, although their occurrence is infrequent throughout S $\gamma$ 4. Such a comparison had heretofore been impossible for any species because of the lack of a complete sequence for the donor switch site, S $\mu$ . They have also carried out intraspecies and interspecies comparisons of the available sequences for S $\mu$  and S $\gamma$  for human and mouse. From an examination of these comparisons they argue that it is unlikely that the switch sequences are substrates for the homologous recombination machinery.

Finally Drs. Camerini-Otero and Proia and colleagues have examined the role that glycosylation plays in the expression of CD4, a 55kD monomeric membrane-bound glycoprotein expressed on the surface of T helper cells. This protein is a member of the immunoglobulin supergene

family, mediates helper T cell activation and is also the receptor for the human immunodeficiency virus (HIV). To investigate the effect of glycosylation they have created a series of mutant CD4 cDNAs by site-specific mutagenesis that delete one, the other or both of the potential glycosylation sites. By *in vitro* transcription and translation they have confirmed that both potential glycosylation sites are utilized. They also have demonstrated by fluorescence-activated cell sorting (FACS) analysis that oligosaccharide deficient CD4 proteins are expressed poorly or not at all at the cell surface and that glycosylation at the carboxyl terminal site appears to be critical for protein expression. In addition, they have shown that cells transfected with CD4 cDNA with the carboxyl glycosylation site eliminated do not produce immunoprecipitable protein suggesting that such proteins may be structurally abnormal and rapidly degraded.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52008-11 GBB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Expression and Human Genetics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. D. Camerini-Otero	Chief	GBB, NIDDK
	P. Hsieh	Expert	GBB, NIDDK
	C. S. Camerini-Otero	Expert	GBB, NIDDK
	F. Mills	Senior Staff Fellow	GBB, NIDDK
	R. Kiyama	Visiting Fellow	GBB, NIDDK
	R. Gardner	Clinical Associate	GBB, NIDDK
	E. Angov	IRTA Fellow	GBB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Genetics and Biochemistry Branch

## SECTION

Molecular Genetics Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.0

## PROFESSIONAL:

5.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to dissect the biochemical steps involved in genetic recombination we have chosen to focus on a key early step: strand exchange between homologous parental DNAs. The product of this strand exchange reaction is a joint molecule composed of a single-strand circle joined to one end of a linear duplex. Three proteins responsible for this step have been purified: uvsX from phage T4; RecA from *E. coli*; and rec1 from *U. maydis*.

Over the last few years we have reported the partial purification and characterization of similar strand-exchange proteins or recombinases from nuclear extracts of human cells and tissues and embryos of *D. melanogaster*. We have shown that the structure of the protein-free intermediate in strand-exchange is most likely that of a three-stranded DNA. Recently, we have built a model of the precise hydrogen bonding interactions between the third strand and the duplex. Critical tests of the model are now possible; e.g., chemical probing (footprinting) of reactive groups in the third strand. In addition, we have searched for eukaryotic proteins other than recombinases that might either form or bind to three-stranded or triplex DNA. We have purified and characterized a protein from HeLa cells that binds to the TAT triplex. We used a gel shift assay to detect the protein. The protein is greatly enriched by passage through a triplex DNA-affinity column.

Finally, in order to assess the role that homologous recombination might play in immunoglobulin heavy chain class switching, we have sequenced several human switch recombination sites. From an examination of these sequences, we have argued that they probably are not substrates for the homologous recombination machinery.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52011-06 GBB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Toxins and DNA Repair in *Xenopus* Oocytes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Eric Ackerman	Senior Staff Fellow	GBB, NIDDK
Others:	Shailendra Saxena	Visiting Associate	GBB, NIDDK
	Jitendra Saxena	Visiting Associate	GBB, NIDDK
	Timothy M. Jenkins	Visiting Fellow	GBB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Genetics and Biochemistry Branch

## SECTION

Molecular Genetics Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.75

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I. We showed that the toxins  $\alpha$ -sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA. All of these toxins were microinjected into *Xenopus* oocytes. Microinjection of deoxyoligonucleotides, ribonucleotides and ribozymes complementary to the same region of 28S RNA abolishes protein synthesis.

II. During early development *Xenopus* replicates its DNA nearly as fast as *E. coli* in log phase; perhaps indicating that oocytes may be an excellent source of DNA repair activity. We have investigated pyrimidine dimer repair by microinjecting UV-irradiated DNA into oocytes. Repair is demonstrated by the absence of pyrimidine dimers using UV-endonuclease and denaturing agarose gels. We have also studied DNA repair for alkylated and chemically modified DNA.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52012-06 GBB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure-Function Relationships of Lysosomal Enzymes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Richard L. Proia	Research Biologist	GBB, NIDDK
Others:	Debra Boles	IRTA	GBB, NIDDK
	Sukumar Medda	Senior Staff Fellow	GBB, NIDDK
	Ruth Navon	Visiting Associate	GBB, NIDDK
	Shoji Yamanaka	Visiting Fellow	GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.75

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I. The Gly269 → Ser mutation in the β-hexosaminidase α-subunit has been found in homozygous form in 3 non-Jewish patients with adult GM2 gangliosidosis. This is in contrast to the situation in Ashkenazi Jewish patients where the Gly269 → Ser mutation is present but always in compound heterozygosity with a null Tay-Sachs allele.

II. We have isolated near full length cDNAs encoding α and β-subunits of murine β-hexosaminidase. The nucleotide sequence of the α-subunit has been determined. These cDNAs have been used to isolate genomic clones encompassing portions of the murine α and β-subunit genes.

III. The human α and β-subunits have been incorporated into baculovirus vectors which have been used to overexpress β-hexosaminidase B (a β-subunit dimer) and β-hexosaminidase S (an α-subunit dimer). The enzymes have been purified from culture medium of recombinant virus-infected SF9 cells by Concanavalin A affinity chromatography followed by Mono S column chromatography, for the B isozyme, or phenyl-Superose column chromatography for the S isozyme.

IV. A rat endoplasmic reticulum carboxyl esterase has been cloned and sequenced. Other partial cDNAs from this gene family have also been sequenced. The full-length cDNA and mutated versions have been expressed in COS 1 cells in order to identify the protein determinant that signifies retention in the endoplasmic reticulum.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52014-03 GBB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

CD4 Receptor Structure/Function Project

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Daniel Camerini-Otero

Chief

GBB, NIDDK

Others: Richard L. Proia

Research Biologist

GBB, NIDDK

Cynthia Tiff

Clinical Associate

GBB, NIDDK

## COOPERATING UNITS (if any)

## LAB BRANCH

Genetics and Biochemistry Branch

## SECTION

Molecular Genetics Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

1.5

## PROFESSIONAL

1.4

## OTHER

0.1

## CHECK APPROPRIATE BOXES:

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unbolded type. Do not exceed the space provided.)

CD4, a 55KD monomeric membrane-bound glycoprotein expressed on the surface of T helper cells, mediates helper T cell activation and is also the receptor for the human immunodeficiency virus (HIV). The extracellular segment forms four VJ-like regions (V1-V4). Two potential N-linked glycosylation sites are present: one at the carboxyl end of V3 and the other near the amino end of V4.

Previous work has suggested that glycosylation of CD4 is necessary for cell surface expression of the receptor. To clarify the effect of glycosylation on CD4 expression we have created a series of mutant CD4 cDNA's by site-specific mutagenesis which delete one, the other or both potential glycosylation sites. By in vitro transcription and translation we have confirmed that both potential glycosylation sites of CD4 are utilized. We have demonstrated by fluorescence-activated cell sorting (FACS) analysis that oligosaccharide deficient CD4 proteins are expressed poorly or not at all at the cell surface and that glycosylation at the carboxyl terminal site appears to be critical for protein expression. Finally, we have shown that cells transfected with CD4 cDNA with the carboxyl glycosylation site eliminated produce no immunoprecipitable protein suggesting that such proteins may be structurally abnormal and rapidly degraded.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52015-02 GBB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Studies of Protein-DNA Interactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Hsieh	Expert	GBB, NIDDK
Others:	R. Daniel Camerini-Otero	Chief	GBB, NIDDK
	Carol Camerini-Otero	Expert	GBB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Genetics and Biochemistry Branch

## SECTION

Molecular Genetics Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.6

## PROFESSIONAL:

1.5

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have studied early steps in homologous recombination carried out by a human recombinase activity isolated from nuclear extracts of HeLa cells and by *E. coli* recA protein. These early steps involve the recognition by recombinases of DNA sequence homology residing on two DNA molecules and the subsequent pairing of these sequences resulting in joint molecule formation.

We have localized pairing interactions to short, defined regions of DNA <60 bp in length and have isolated stable structures free of any recombinase protein. These joint molecules have unusual thermal stabilities indicating that they are three-stranded DNA structures. Preliminary experiments indicate that a novel triple-stranded DNA in which the third strand is hydrogen-bonded to an intact duplex DNA may be an intermediate in homologous recombination.

We are also examining the effects of DNA mismatches, deletions and insertions on nonenzymatic branch migration. Branch migration involves the exchange of hydrogen bonds between two identical DNA strands and is thought to play an important role in homologous recombination. We have designed a convenient and sensitive method for monitoring branch migration through short, defined sequences in order to study the effect of sequence context on branch migration.

Annual Report of The Digestive Diseases Branch  
National Institute of Diabetes, and Digestive and Kidney Diseases

SUMMARY OF BRANCH ACTIVITIES

During this past year the Digestive Diseases Branch added a new section resulting in a total of four sections in the Branch - Section on Gastroenterology, Section on Cell Biology, Liver Diseases Section and Section on Clinical Investigation (new section). Detailed summaries of the activities of each section follow. All four sections are engaged in investigations of basic biologic processes (e.g., hormone action, membrane transport, cellular and humoral immunology) and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver, pancreas and gastrointestinal tract. All four sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, hepatitis and fulminant hepatic failure.

SECTION ON GASTROENTEROLOGY

Jean Martinez of Montpellier, France synthesized an analogue of the C-terminal heptapeptide of cholecystokinin (CCK) that we have found to be particularly useful for examining receptors for CCK on pancreatic acinar cells. Pancreatic acinar cells possess two classes of receptors that interact with CCK - one class has a high affinity for CCK and mediates maximal stimulation of enzyme secretion; the other class has a low affinity for CCK and mediates submaximal stimulation of enzyme secretion. The Martinez analogue (CCK-JMV-180) possesses the interesting properties of being a full agonist at high affinity CCK receptors and a competitive antagonist at low affinity CCK receptors.

Until recently it was believed that mobilization of cellular calcium mediated the action of CCK on high affinity CCK receptors on pancreatic acinar cells. Using CCK-JMV-180 we have shown that mobilization of cellular calcium is associated with occupation of low affinity CCK receptors on pancreatic acinar cells. The mediator of the action of CCK at high affinity CCK receptors on pancreatic acinar cells remains to be determined.

Like CCK, carbachol, a muscarinic cholinergic agonist that is resistant to degradation by abolinesterase, also modifies the properties of receptors for other secretagogues on pancreatic acinar cells. Pancreatic acinar cells possess two classes of receptors that interact with VIP - one class has a high affinity for VIP and mediates stimulation of enzyme secretion; the other has a low affinity for VIP. Carbachol causes a 30- to 50-percent decrease in the number of high affinity VIP receptors with no change in the number of low affinity VIP receptors or in the affinities for VIP of either class of receptors. Because pancreatic acinar cells possess so many "spare" high affinity VIP receptors, the carbachol-induced decrease in the number of high affinity VIP receptors is not sufficient to reduce VIP-stimulated enzyme secretion.

Carbachol also modifies receptors for secretagogues other than VIP on pancreatic acinar cells. Pancreatic acinar cells possess two classes of cholinergic receptors that interact with carbachol - one class has a high affinity for carbachol; the other has a low affinity for carbachol. Occupation of the high affinity cholinergic receptors by carbachol reduces the number of high affinity cholinergic receptors but does not alter the number of low affinity receptors. Occupation of low affinity cholinergic receptors by carbachol does not alter the number or affinity of either high affinity or low affinity cholinergic receptors, reduces the number of bombesin receptors, reduces the number of high affinity CCK receptors and does not alter the low affinity CCK receptors. These carbachol-induced changes in receptor number are accompanied by a reduction in stimulation of enzyme secretion by the appropriate secretagogue.

In a number of different cell systems radiolabeled cholecystokinin ( $^{125}\text{I}$ -CCK-8) and radiolabeled gastrin ( $^{125}\text{I}$ -gastrin) have been used to measure binding of CCK-8 and gastrin to their cell surface receptors. In a recent study using gastric chief cells, however, we showed that although  $^{125}\text{I}$ -CCK-8 and  $^{125}\text{I}$ -gastrin bind to the cells, the binding sites are not the receptors with which CCK and gastrin interact to alter chief cell function. This actively illustrates the degree of caution that investigators must exercise in correctly interpreting radioligand binding studies.

#### SUMMARY OF SECTION ON CELL BIOLOGY

##### I. Studies relating to the identification and characterization of receptors for gastrointestinal peptides.

###### A. Development of bombesin (Bn) receptor antagonists.

In previous studies we have discovered, in collaboration with Dr. D.H. Coy (Tulane University, School of Medicine, Peptide Research Lab) and Biomeasure Corporation, Hopkinton, MA., four different classes of bombesin receptor antagonists. During the last year it has been possible to develop even more potent Bn receptor antagonists which have antagonist activity in all species examined.

Studies of both Bn and the structurally-related peptide litorin demonstrated that the COOH-terminal amino acid of both peptides is important for initiating a biological response but not essential for determining receptor affinity. Structure-function studies of des Met<sup>14</sup> Bn analogues demonstrated that both the amide and alkylamide were potent antagonists of the action of Bn on 3T3 cells or guinea pig pancreatic acini. The affinity depended on the chain length of the alkyl moiety (R) added to the analogue [D-Phe<sup>6</sup>]Bn(6-13)NH<sub>2</sub> with propyl > ethyl > H. The replacement of the amide with a free carboxyl group significantly decreased affinity. The results suggested that the position 13-CO group of Bn, at least in des Met<sup>14</sup> analogues, might be involved in binding to the Bn/GRP receptor via hydrogen binding and thus affinity would be enhanced by electron-releasing alkyl substitutions and decreased by a free carboxyl group in that the CO electrons are distributed over two O groups.

Additional structure-function studies of various des Met<sup>14</sup> Bn analogues demonstrated that the insertion of certain D-amino acids in the 6 position of Bn from the COOH-terminus, such as [D-Phe<sup>6</sup>], resulted in truncated Bn(6-13) analogues which had equal or greater affinity than Bn(1-13) analogues for the Bn receptor. [D-Phe<sup>6</sup>]Bn(6-13) propylamide was the most antagonist found in this study and had equal affinity for the Bn receptor to GRP or Bn itself. Some of the Bn analogues such as [D-Phe<sup>6</sup>]Bn(6-13) propylamide were shown to also be potent antagonists in vivo and because it offers fewer proteolytic degradation sites and is a relatively short peptide, should be useful for in vivo studies.

Previous studies demonstrated that pseudopeptide analogues of either the COOH-terminus of Bn or the structurally-related peptides, gastrin-releasing peptide (GRP) or litorin were another class of potent Bn receptor antagonists in 3T3 cells or guinea pig pancreatic acini. Recent studies suggested some of these analogues might have partial agonist activity in other species. In detailed structure-function studies using rat pancreatic acini which possess a Bn receptor which has much less stringent requirements for Bn receptor activation than that on 3T3 cells or guinea pig pancreatic acini, the ability of various pseudopeptide analogues of Bn-related peptides (i.e. with the -CONH- bond in position 13 reduced to -CH<sub>2</sub>NH-) to initiate a biologic response was investigated. A number of the pseudopeptides which were potent Bn antagonists in 3T3 cells or guinea pig pancreas had agonist activity in the rat pancreas; however, the insertion of either a D-Phe<sup>14</sup> or p-chloro Phe<sup>14</sup> resulted in potent antagonists in all species. Therefore, peptides such as [D-Phe<sup>6,14</sup>,  $\psi$  13-14]Bn(6-14) or [D-Phe<sup>6</sup>,cpa<sup>14</sup>]Bn(6-14) were discovered to be potent antagonists in all species examined and thus might also be useful for in vivo studies.

B. Development and/or characterization of receptor antagonists for other gastrointestinal peptides.

In collaboration with Dr. D.H. Coy (Tulane University) a novel class of substance P receptor antagonists has been identified which has enhanced specificity for substance P receptors. One analogue, Leu<sup>10</sup>  $\psi$ (CH<sub>2</sub>NH)Leu<sup>11</sup>-substance P had greater than 50 times high affinity for substance P than for bombesin receptors. In comparison [D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>]-substance P, which is a currently, widely used substance P receptor antagonist, has only a 1.5-fold higher affinity for substance P receptors than bombesin receptors.

C. Identification and characterization of receptors for gastrointestinal hormones using various radioligands.

Recent studies by our laboratory and in collaboration with Dr. Severi in Rome have provided evidence that similar to receptors mediating the action of a number of other gastrointestinal peptides, more than one subtype of Bn receptor exists. On both rat pancreatic acini and guinea pig gastric smooth muscle cells, a Bn receptor with high affinity for GRP, bombesin and neuromedin C and low affinity for neuromedin B (GRP-preferring) was identified by using binding studies or functional assays with various antagonists. On

rat esophageal muscularis mucosa and isolated gastric smooth muscle cells from the guinea pig a Bn-receptor with greater than a 100-fold higher affinity for NMB than GRP or neuromedin C was identified by autoradiographic studies, binding studies or functional assays using various agonists and antagonists. Recent collaborative studies with Dr. Terry W. Moody, Biochemistry Department, Georgetown University and Dr. Tim Moran, Department of Psychiatry, Johns Hopkins University, have provided evidence that at least two subtypes of Bn receptors also are present in the central nervous system. Similar to gastrointestinal tissues, one type of Bn receptor is neuromedin B-preferring and is the most prevalent Bn receptor in the CNS, whereas the other type Bn receptor is GRP-preferring with preliminary studies suggesting primarily this type of Bn receptor in the nucleus accumbens and olivary nucleus in the hindbrain.

We have discovered that the four different classes of Bn receptor antagonists have markedly different affinities for the two Bn receptor subtypes. The antagonists [D-Phe<sup>6</sup>]Bn(6-13) ethyl ester, [D-Phe<sup>6</sup>,Cpa<sup>14</sup>,ψ13-14]Bn(6-14), Leu<sup>14</sup>,ψ13-14]Bn and [Leu<sup>14</sup>,ψ9,10]Bn have a 10,000-, 1425-, 122- and 40-fold, respectively, higher affinity for Bn receptors that were GRP- or NMC-preferring than Bn receptors that were neuromedin B-preferring. In contrast, the antagonists [Tyr<sup>4</sup>,D-Phe<sup>12</sup>]Bn, [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>,Leu<sup>11</sup>]SP-4-11 and [D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>]SP had a 4-, 7- and 9-fold respectively, higher affinity for Bn receptors that were neuromedin B-preferring than Bn receptors which were GRP-preferring. Some of these antagonists have been shown to be active in vivo, and thus will allow the subtype of Bn receptor mediating different physiological processes to be differentiated.

## II. Studies related to mechanism of the ability of gastrointestinal peptides to alter cellular function.

In previous studies we and others have shown that cholecystokinin (CCK), interacts with specific high affinity receptors on pancreatic acinar cells, activates phospholipase C, stimulates increases in phosphoinositides and mobilizes cellular calcium which results in enzyme secretion. To gain additional insight into the ability of CCK to alter cell function, the kinetics and stoichiometry of CCK and a related peptide, CCK-JMV-180 [Boc-Nle<sup>28,31</sup>,CCK(27-32)-2 phenylethyl ester], an analogue of CCK with partial agonist activity to stimulate the accumulation of inositol phosphates, mobilize cellular calcium and stimulate enzyme secretion were compared. CCK-8 caused a rapid increase in [<sup>3</sup>H]IP<sub>2</sub> and [<sup>3</sup>H]IP<sub>3</sub> (2-fold over basal at 10 sec), and HPLC analysis demonstrated at 10 sec, 100% of the [<sup>3</sup>H]IP<sub>3</sub> was IP<sub>3</sub>(1,4,5). CCK-JMV-180 caused no increase in [<sup>3</sup>H]IP<sub>3</sub> at 10 sec; however, by a sensitive radioreceptor mass assay a 3-fold increase was detected. Each peptide mobilized cellular Ca, although CCK-JMV-180 was only 45% as effective as CCK. Comparison of the abilities of each peptide to stimulate IP<sub>3</sub>(1,4,5) accumulation to mobilize cellular Ca and stimulate enzyme secretion demonstrated that CCK-related peptides cause maximal enzyme secretion with minimal changes in calcium mobilization and maximal changes in calcium mobilization with minimal changes in IP<sub>3</sub>(1,4,5), suggesting marked amplification.

We investigated the importance of sulfation of gastrin or CCK on influencing their affinity for gastrin or CCK receptors by comparing the abilities of sulfated gastrin-17 (gastrin-17-II), desulfated gastrin-17 (gastrin-17-I), CCK-8 and desulfated CCK-8 [des(SO<sub>3</sub>)CCK-8] to interact with CCK or gastrin receptors on guinea pig pancreatic acini. For inhibiting binding of <sup>125</sup>I-gastrin to gastrin receptors the relative potencies were: gastrin-17-II > CCK-8 > gastrin-17-I > des(SO<sub>3</sub>)CCK-8. For inhibiting binding of <sup>125</sup>I-Bolton Hunter-labeled CCK-8 to CCK receptors the relative potencies were: CCK-8 >> des(SO<sub>3</sub>)CCK-8 = gastrin-17-II > gastrin-17-I. These results demonstrate that, in contrast to older studies, sulfation of both CCK and gastrin increase their affinities for both gastrin and CCK receptors. Moreover, the gastrin receptor is relatively insensitive to the position of the sulfate moiety, whereas the CCK receptor is extremely sensitive to both the presence and exact position of the sulfate moiety.

In recent studies we and others have shown that both CCK and gastrin receptors can occur on the same cell. Furthermore, CCK-related peptides have been demonstrated in most cells to bind to two different classes of binding sites. To characterize the ability of CCK and related peptides to interact with binding sites on pancreatic acini and alter cell function, we used various CCK and gastrin receptor agonists and antagonists which have differing affinities for these two receptors. For inhibition of binding of <sup>125</sup>I-BH-CCK-8 to guinea pig pancreatic acini, the potencies for agonists were CCK-8 > desulfated [des(SO<sub>3</sub>)] CCK-8 > gastrin-17-I > pentagastrin > CCK-4 and for the antagonists, L-364,718 > proglumide analogue 10 > CBZ-CCK-(27--32)-NH<sub>2</sub>. For all nonsulfated agonists, the curves were biphasic with 20% of the tracer bound to sites with high affinity for these agonists with the following relative potencies: gastrin-17-I > pentagastrin > des(SO<sub>3</sub>)CCK-8 >> CCK-4; whereas 80% was bound to low affinity sites with the following potencies: des(SO<sub>3</sub>)CCK-8 > gastrin-17-I = pentagastrin >> CCK-4. For L-364,718 and proglumide analogue 10, 80% of <sup>125</sup>I-BH-CCK-8 was bound to sites with high affinity for these antagonists and 20% to sites with low affinity. Computer analysis revealed that the dose-inhibition curve for gastrin-17-I was significantly better fit by a three-site model than by a two-site model. These results demonstrate that pancreatic acinar cells possess three classes of CCK receptors, not two as reported previously. Two classes are CCK-preferring, binding 83% of <sup>125</sup>I-BH-CCK-8 at tracer concentrations, and comprise high- and low-affinity CCK-preferring sites that can be distinguished by all agonists, but have equally high affinity for L-364,718 and proglumide 10. A third class binds 17% of the tracer, cannot be differentiated from high-affinity CCK-preferring receptors by CCK-8, and has low affinities for L-364,718 and proglumide 10.

Recently we developed a tissue section method which allowed us to perform binding to small pieces of tissues and investigate the localization using autoradiography. One of the primary physiological functions of the hormone, CCK, is to influence the contractile state of the sphincter of Oddi (SO). To characterize directly the ability of CCK to interact with specific receptors on the SO, we measured binding of <sup>125</sup>I-BH-CCK-8 to tissue sections from the



guinea pig SO. Autoradiography localized binding of  $^{125}\text{I}$ -BH-CCK-8 over the smooth muscle layer of the sphincter. Binding of  $^{125}\text{I}$ -BH-CCK-8 to SO tissue sections was saturable, specific, dependent on time, pH and temperature, and was reversible. Binding of  $^{125}\text{I}$ -BH-CCK-8 was inhibited by various CCK receptor agonists with the following potencies: CCK-8 > des(SO<sub>3</sub>)CCK-8 > gastrin-17-I and by various CCK receptor antagonists with the following potencies: L-364,718 > proglumide analogue 10 > CBZ-CCK-27-32-NH<sub>2</sub> > Bt<sub>2</sub>cGMP. Analysis of binding of  $^{125}\text{I}$ -BH-CCK-8 to SO tissue sections revealed two classes of CCK binding sites: a high affinity site ( $K_d$  0.2nM), and a low affinity site ( $K_d$  70nM). Atropine (0.1μM) or tetrodotoxin (1μM) caused a similar rightward shift of the CCK-8-dose-response curve for stimulation of contraction of the SO. Comparison of receptor occupation to CCK-8-induced contraction suggested that the high affinity binding site correlated with the ability of CCK-8 to cause contraction in the presence of atropine or tetrodotoxin. These results suggest the possibility that high affinity CCK binding is to neural elements which mediates atropine- and tetrodotoxin-sensitive SO contraction, whereas low affinity binding is to muscle mediates atropine- and tetrodotoxin-insensitive SO contraction.

Recent studies suggest that the COOH terminal amino acid of Bn or GRP is crucial for initiating a biologic response but not for determining affinity. In collaboration with Dr. David H. Coy, Peptide Research Labs, Tulane University, we examined the important structural determinants in Bn-related peptides for determining Bn receptor affinity in murine 3T3 cells, rat and guinea pig pancreatic acini and the ability to initiate biologic responses by synthesizing 18 des Met<sup>14</sup> Bn(6-13) analogues. With both guinea pig acini and 3T3 cells, affinity was affected by the chain length of the alkyl moiety (R) added to [D-Phe<sup>6</sup>]Bn(6-13)NH<sub>2</sub>R whereas in rat acini affinity was not. The affinity in all 3 cell systems was increased by additions of other electron releasing groups to the COOH-terminal carboxyl group such as [D-Phe<sup>6</sup>]Bn(6-13)ethyl or methyl ester or hydrazide. Comparison of the ability of the various analogues to alter biologic activity in guinea pig pancreas and 3T3 cells revealed that 12 were antagonists, 1 a full agonist and 5 were partial agonists. In rat acini 8 were antagonists, 5 full agonists and 5 partial agonists. Potent antagonists in each cell system were the methyl and ethyl ester, hydrazide and ethylamide analogues. In 3T3 cells or guinea pig pancreas, agonist activity of the alkylamide was critically dependent on the chain length, whereas with rat pancreatic Bn receptors any alkylamide longer than the ethylamide had agonist activity. These results demonstrate that the nature of the substitution on the carboxyl terminal of des Met<sup>14</sup> Bn analogues is critically important, not only for determining Bn receptor affinity but also for determining the ability to initiate a biologic response. In contrast to previous studies, the present results demonstrate that the presence of the COOH terminal amino acid in position 14 of Bn is not essential for initiating a biologic response.

## SECTION OF CLINICAL INVESTIGATION

### I. Studies in patients with Zollinger-Ellison syndrome.

The Clinical Investigation Section of the Digestive Diseases Branch has cared for about 170 patients with Zollinger-Ellison syndrome (ZES, a gastrin-producing tumor associated with hypergastrinemia and gastric acid hypersecretion), and smaller numbers of patients with other islet cell tumors.

A prospective study of the use of oral omeprazole in 40 patients with ZES treated for up to 5 years established that the drug was highly effective in symptom relief and mucosal healing in these patients. Omeprazole was more convenient than oral histamine  $H_2$  receptor antagonists and more effective. No significant toxicity of omeprazole was noted clinically or biochemically. The drug did not produce any apparent changes in the stomach.

A prospective study of the standard meal test in patients with proven Zollinger-Ellison syndrome defined the utility of the test which has been suggested as being useful in differentiating between ZES and G-cell hyperplasia. The study found that 16% of patients met the criteria previously described for G-cell hyperplasia, and thus this test does not differentiate between this condition and ZES.

A prospective study of the secretion and calcium infusion tests, described previously as being useful for the diagnosis of ZES, established the usefulness of these tests. The secretion test proved positive in 85% of 80 patients using the criterion of a rise in plasma gastrin of  $>200$  pg/ml, with the early time points after injection being most useful. The calcium infusion test was positive in only 53% of patients but was positive in 33% of patients with a negative secretion test. Thus the calcium test may be of use in difficult cases.

A series of 9 patients with MEN-1 or MEN-2 and islet cell tumors underwent surgery. These patients with MEN-1 with insulinoma and one with VIPoma were cured by local resection of tumor, but 6 patients with MEN-1 and ZES were not cured. However, the patient with MEN-2 and ZES was cured of this tumor. Thus, gastrinomas in MEN-1 differ from other islet cell tumors in MEN-1 and gastrinomas in MEN-1.

A study of reflux esophagitis and its response to therapy in 122 patients with ZES demonstrated that reflux was not rare, as had been thought previously, but occurred at some time in 61% of patients. When acid outputs were reduced to  $<10$  mEq/h, reflux disease resolved in 2/3 of those affected. However, the other 33% required acid to be lowered to  $<5$  or  $<1$  mEq/h to resolve the disease completely.

In 20 patients with ZES undergoing surgery, acid output was controlled using I.V. omeprazole given as a bolus of 60 mg every 12 hrs. In 19 of the 20 patients acid output was controlled to  $<10$  mEq/h, but the other patient required 100 mg/ql2h. I.V. omeprazole produced no significant side effects in treatment periods of up to 18 days and should be useful for ZES patients who are unable to take oral medication.

## II. Studies of gastric smooth muscle cells.

We have previously described that calcitonin gene-related peptide (CGRP), a peptide found in nerves in the gastrointestinal tract and elsewhere, causes relaxation of gastric smooth muscle cells, through occupation of specific receptors on the cells. Recent studies established that the related peptide salmon calcitonin is a partial agonist at this receptor, but the C-terminal peptide of CGRP, [Tyr<sup>0</sup>]CGRP-28-37 acts as an antagonist of CGRP, while shorter C-terminal peptides of CGRP have no actions at the CGRP receptor, but act as agonists at the CCK receptor.

Gastric smooth muscle cells relax on exposure to  $\beta$ -adrenergic agonists. We have demonstrated that gastric smooth muscle cells possess exclusively  $\beta_2$ -adrenergic receptors, and relaxation, whether due to CGRP or occupation of  $\beta_2$ -receptors, is associated with an increase in cellular cyclic AMP.

## Liver Diseases Section

The Liver Diseases Section is currently responsible for seven principal projects.

### I. Studies Relating to the Pathogenesis of Hepatic Encephalopathy

The abnormal pattern of visual evoked responses (VERs) in animals with hepatic encephalopathy (HE) due to fulminant hepatic failure (FHF) resembles that induced by drugs which promote GABAergic neurotransmission, including benzodiazepines (BZs). Furthermore, animals with HE due to FHF exhibit increased resistance to drugs that induce convulsions by decreasing GABAergic tone. Ameliorations of HE (both clinical and electrophysiologic) have been induced in animals with FHF by BZ receptor antagonists. Furthermore, Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABAergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE. Autoradiographic and neurochemical evidence for the existence of such a ligand has been found in the brains of animal models of HE. Recently several known 1,4 BZs have been shown to be present in normal brain and to be present in increased concentrations in the brains of animals and humans with FHF. [E.A. Jones, J. Vergalla, S.H. Gammal, B.L. Baker, A.S. Basile, P. Skolnick].

### II. Trials of Therapies for Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative, presumably autoimmune, destruction of small bile ducts. As some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with PBC. In a randomized controlled trial of chlorambucil therapy, which involved 24 patients, treatment with the drug was associated with a decrease in the rate of increase in serum bilirubin, normalization of elevated serum IgM levels, an improvement in inflammatory cell infiltrate in the liver and a variable degree of bone marrow suppression. These findings strongly suggest that immunosuppressive therapy can retard the progression of PBC. More effective less toxic immunosuppressive regimens are being sought for this disease. Currently the effects of low-dose methotrexate are being evaluated in an open trial. [E.A. Jones, J.H. Hoofnagle, N.V. Bergasa, J. Korenman, T.L. Fong, A.M. DiBisceglie].

### III. Studies of Cellular Immune Function in Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the mediation of the hepatobiliary lesion of primary biliary cirrhosis (PBC) is being studied. Recently, with the use of monoclonal antibodies, it has become apparent that CD4 (T4) T cells can be subdivided into subpopulations having unique functions. In particular CD4 positive, Leu-8 positive T cells have been demonstrated to have direct suppressor function, as well as the capacity for inducing CD8 (T8) suppressor cells. In addition, it has been shown that the CD4 positive, Leu-8 positive T cell population is the predominant autoreactive T cell subpopulation in peripheral blood. CD4+, Leu-8+ T cells from patients with PBC, but not from patients with other liver diseases, have

been shown to exhibit a defect in their ability to suppress immunoglobulin synthesis by B cells in vitro. Furthermore the proliferative responses of these cells from patients with PBC to mitogenic stimulation was found to be impaired. The abnormal function of the CD4+, Leu-8+ T cell subpopulation in patients with PBC may play a central role in the defective immunoregulation found in this disease. Exposure of this subpopulation of T cells from patients with PBC to phorbol ester, which activates the protein kinase C pathway, corrects their abnormal function. Thus a defect in the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. [E.A. Jones, R. Moreno-Otero, S.P. James, J.H. Hoofnagle, J. Vergalla].

#### IV. Studies of the Natural History and Treatment of Chronic Type B Hepatitis

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials in which antiviral or immunomodulatory agents are being administered. A randomized controlled trial of interferon therapy versus no treatment is underway. Forty four patients have been entered and 31 have completed interferon therapy and followup. Eleven (35%) responded with loss of HBeAg from serum. In addition, a pilot study is examining the effect of one month of corticosteroid pre-treatment followed by interferon therapy for patients who have previously not responded to interferon therapy alone. [J.H. Hoofnagle, A.M. DiBisceglie, P. Martin, N.V. Bergasa, J. Korenman, M. Lisker-Melman, B.L. Baker, T.L. Fong, E.A. Jones; not NIH: J. Gerin, M. Sjogren].

#### V. Studies of the Natural History and Treatment of Chronic Non-A, Non-B (Type C) Hepatitis

Patients with well-documented chronic non-A, non-B hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients is available to enable experimental therapies for this disease to be evaluated. A pilot study demonstrated that alpha interferon was effective in normalizing serum ALT activities in a majority of cases. This effect was associated with an improvement in liver histopathology (decreased activity of hepatitis). A prospective randomized, placebo-controlled trial of alpha interferon therapy for chronic non A, non B hepatitis has been completed. Forty one patients have completed the initial 6 months blinded phase. Eighteen patients have been crossed over from placebo to receive interferon for 12 months. Interferon appears to be effective in decreasing serum aminotransferase activities and improving liver histopathology in the majority of cases although the disease relapses in a substantial proportion of patients after stopping therapy. [J.H. Hoofnagle, A.M. Di Bisceglie, N.V. Bergasa, J. Korenman, H.J. Alter, J. Everhart, E.A. Jones; not NIH: Z. Goodman, G. Kuo].

#### VI. Studies of the Natural History and Treatment of Duck Hepatitis B Virus Infection

Duck hepatitis B virus (DHBV) infection is a potentially useful experimental model of human hepatitis B virus infection. The ability of new antiviral and immunomodulatory agents to suppress DHBV replication in ducks is being assessed. It is anticipated that the ability of a drug to suppress DHBV replication will be shown to be a satisfactory screening test for new effective therapies for chronic type B hepatitis in man. Reproducible assays for quantitating DHBV DNA and DNA polymerase in serum have been established. 2',3'-dideoxynucleosides (ddC, ddA, ddG,

ddl) have been shown to be potent inhibitors of DHBV replication and ddl does not appear to cause severe side-effects. A longterm therapeutic trial (2 months) of ddG has shown that this agent induces a decrease in serum levels of DHBV DNA and DNA polymerase, while causing little change in hepatic levels of DHBV DNA. The effect of ddl will shortly be investigated in humans with hepatitis B infection. [E.A. Jones, P. Martin, H. Robcis, R. Miller, A.M. Di Bisceglie, J. Korenman, H. Mitsuya, S. Broder, J.H. Hoofnagle].

## VII Studies of the Opiate System in Cholestatic Liver Disease

Pruritus is a common distressing complication of cholestatic liver diseases. The hypothesis that endogenous opiate ligands may contribute to this symptom is suggested by the precipitation of an opiate withdrawal-like syndrome by an opiate antagonist in patients with chronic cholestatic liver diseases and the tendency of exogenously administered opiates to induce pruritus. To test this hypothesis the effects of infusing naloxone in eight pruritic patients with primary biliary cirrhosis were assessed using a newly designed device which enables scratching activity to be continuously recorded independent of coarse body movements. Naloxone reduced scratching activity substantially in each patient. Further studies of the opiate system in cholestasis revealed that bile duct ligation in the rat induces a state of mild analgesia which can be reversed by naloxone. These findings suggest that cholestasis is associated with increased opiate tone and that long term amelioration of the pruritus that complicates this syndrome may be possible by administering an opiate antagonist that is effective when given orally. [E.A. Jones, N.V. Bergasa, T.L. Fong, M. Fried, M.G. Swain, D. Alling, T. Talbot, W. Schmidt].

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53001-20 DDB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Membrane Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Jerry D. Gardner	Chief	DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Sec.	DDB, NIDDK
	P.N. Maton	Visiting Scientist	DDB, NIDDK
	S.A. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	H.C.V. Chiang, I. Waxman	Medical Staff Fellows	DDB, NIDDK
	L. Zhang, D. Menozzi, R. Amin	Visiting Fellows	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	R. Patto	Special Volunteer	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

3.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53002-18 DDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Gastrointestinal Hormones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Gardner	Chief	DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Sec.	DDB, NIDDK
	P.N. Maton	Visiting Scientist	DDB, NIDDK
	S. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	H.C.V. Chiang, I. Waxman	Medical Staff Fellows	DDB, NIDDK
	S-C. Huang, D. Menozzi, L. Zhang	Visiting Fellows	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	R. Patto	Special Volunteer	DDB, NIDDK

## COOPERATING UNITS (# any)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Section on Gastroenterology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

3.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin, bombesin, substance P and vasoactive intestinal peptide with their specific membrane receptors.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53004-18 DDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cyclic Nucleotide Mediated Functions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Gardner	Chief	DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Sec.	DDB, NIDDK
	P.N. Maton	Visiting Scientist	DDB, NIDDK
	S.A. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	H.C.V. Chiang, I. Waxman	Medical Staff Fellows	DDB, NIDDK
	L. Zhang, D. Menozzi, R. Amin	Visiting Fellows	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	R. Patto	Special Volunteer	DDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Section on Gastroenterology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.2

## PROFESSIONAL:

3.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53100-02 DDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Identification and Characterization of Receptors for GI Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
Others:	Jerry D. Gardner	Chief	DDB, NIDDK
	S. Wank, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	V. Chiang	Medical Staff Fellow	DDB, NIDDK
	J-M Qian, M. Haffar	Visiting Fellows	DDB, NIDDK
	L-H Wang, S-C Huang	Visiting Fellows	DDB, NIDDK
	S. Mantey	Chemist	DDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.3

## PROFESSIONAL:

2.8

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies involve the identification of receptors for gastrointestinal peptides by developing selective radioligands for specific receptors, specific potent antagonists, and the characterization of receptors using these ligands, antagonists, cross-linking studies, solubilization and eventually structural determination.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53101-02 DDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Basis of Action of Gastrointestinal Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
Others:	Jerry D. Gardner	Chief	DDB, NIDDK
	S. Wank, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	V. Chiang	Medical Staff Fellows	DDB, NIDDK
	J-M Qian, M. Haffar	Visiting Fellows	DDB, NIDDK
	L-H Wang, S-C Huang	Visiting Fellows	DDB, NIDDK
	S. Mantey	Chemist	DDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.3

## PROFESSIONAL:

2.8

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project includes studies investigating the relationship for various gastrointestinal peptides between receptor occupation to subsequent changes induced in cellular processes, the characterization of the various second messengers including cyclic nucleotides, changes in cellular calcium, breakdown of phosphoinositides, generation of diacylglycerol as well as characterization of more distal steps in cell activation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53200-01 DDB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Management of Islet Cell Tumors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.N. Maton	Visiting Scientist	DDB, NIDDK
Others:	J.D. Gardner	Chief	DDB, NIDDK
	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	B. Haffar, G. Slimak	Medical Staff Fellows	DDB, NIDDK
	J. O'Brien, I. Waxman	Medical Staff Fellows	DDB, NIDDK
	L. Miller, R. Vinayek	Medical Staff Fellows	DDB, NIDDK
	H. Frucht, V. Chiang	Medical Staff Fellows	DDB, NIDDK

## COOPERATING UNITS (if any)

National Cancer Institute, Surgery Branch	J.A. Norton
Radiology Department, Clinical Center	J.L. Doppman

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Clinical Investigation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.75

## PROFESSIONAL:

2.75

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies involving patients with islet cell tumors (Zollinger-Ellison syndrome, insulinoma, VIPoma) are directed to further understanding the pathogenesis of the syndromes and to developing alternative, more effective modes of therapy utilizing both medical and surgical approaches.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53201 -01 DDB

PERIOD COVERED

October 17 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Receptors on Gastric Smooth Muscle Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. N. Maton	Visiting Scientist	DDB, NIDDK
Others:	J. D. Gardner	Chief, Digestive Diseases Br.	DDB, NIDDK
	R. T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	L. Zhang	Visiting Fellow	DDB, NIDDK
	T. Pradhan	Chemist	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Clinical Investigation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.7

PROFESSIONAL:

1.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In vitro systems are being used to identify receptors on gastric smooth muscle cells by means of radiolabelled ligands, agonists and antagonists, and to examine the mechanisms by which agonists exert their effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53501-17 DDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies Relating to the Pathogenesis of Hepatic Encephalopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	B.L. Baker	Research Associate	LDS, NIDDK
	M.G. Swain	Guest Researcher	LDS, NIDDK
	C. Yurdaydin	Visiting Associate	LDS, NIDDK
	M. Fried	Clinical Associate	LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Neuroscience, NIDDK (P. Skolnick and A.S. Basile)

LAB/BRANCH

Digestive Diseases Section

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Rabbits and rats with HE due to FHF exhibit increased resistance to the convulsive effects of bicuculline (a GABA receptor antagonist) and 3-mercaptopropionic acid (an inhibitor of GABA synthesis), respectively. Both clinical and electrophysiologic (VER waveform) ameliorations of HE, have been induced in animals with FHF by benzodiazepine (BZ) receptor antagonists. Furthermore, spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. In addition a BZ receptor antagonist reversed the hypersensitivity of HE rabbit neurons to depression by a GABA agonist. The functional status of the chloride ionophore of the GABA/BZ receptor complex has been shown to be normal in a rat model of HE due to FHF. Radioligand binding to BZ receptors, determined autoradiographically, was decreased in thin unwashed sections from HE rabbit brains. Purification and characterization of HE rat brain extracts revealed the presence of reversible, competitive, heat and protease stable BZ receptor ligands with agonist properties. Two of these ligands have been chemically characterized as the 1,4 BZs diazepam and N-desmethyldiazepam. The concentrations of these compounds were 2-9 fold greater in HE rat brain than control brain. Overall, these findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) Endogenous BZ receptor agonists probably contribute to HE.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK, 53503-16 DDB

PERIOD COVERED  
October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Immunologic Studies of Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director	DDN, NIDDK
	J. Vergalla	Chemist	LDS, NIDDK
	R. Moreno-Otero	Guest Researcher	LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Clinical Investigation, NIAID (S.P. James)

LAB/BRANCH  
Digestive Diseases Branch

SECTION  
Liver Diseases Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:
-------------------------	----------------------	--------

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects  
☐ (a1) Minors  
☐ (a2) Interviews  
☒ (b) Human tissues  
☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary biliary cirrhosis (PBC) appears to be a model autoimmune disease. Abnormal immune mechanisms are being studied in this disease, but so far a disease-specific immunologic deficit has not been defined with certainty. Recently recognized defects of humoral immunity include: (i) Evidence for the existence of an expanded clone of activated B cells that synthesize mitochondrial antibodies with different antigenic specificities from those synthesized by normal B cells; and (ii) Detection of a serum factor, probably an abnormally immunoreactive IgM, which blocks the binding of C3b-opsonized erythrocytes by monocytes. Recently recognized defects in cellular immunity include: (i) A diminished ability of patient T cells to suppress immunoglobulin synthesis; and (ii) Hyporeactivity of lymphocytes in the autologous mixed lymphocyte reaction, which normally leads to activation of suppressor T cells. To determine whether such abnormalities of lymphocyte function in PBC might be due to altered function of immunoregulatory T cell subpopulations, phenotypic and functional characteristics of T cells that have the CD4 antigen detectable (by monoclonal antibody) on their surface were examined. Patients with PBC were found to have normal numbers of CD4+, Leu-8+ T cells, but, in contrast to patients with other liver diseases, suppression of immunoglobulin synthesis and mitogen-stimulated proliferation mediated by this subpopulation of T cells were defective. These defects may play a central role in the abnormal immunoregulation found in this disease.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53505-15 DDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Alpha-1-Antitrypsin Phenotypes and Metabolism

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.A. Jones Chief LDS, NIDDK

Others: J. Vergalla Chemist LDS, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Liver Diseases Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

0

## PROFESSIONAL

0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
    — (a1) Minors  
    — (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been terminated.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZOI DK 53508-13 DDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Hepatic Receptors for Glycoproteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: E.A. Jones

Chief

LDS, NIDDK

Others: J. Vergalla

Chemist

LDS, NIDDK

## COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism, NIDDK (G. Ashwell)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Liver Diseases Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

0

## PROFESSIONAL

0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53509-12 DDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Type B Hepatitis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.H. Hoofnagle	Director, DDDN	NIDDK
Others:	E.A. Jones	Chief	LDS, NIDDK
	A.M. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	N.V. Bergasa	Medical Staff Fellow	LDS, NIDDK
	J. Korenman	Medical Staff Fellow	LDS, NIDDK
	B.L. Baker	Medical Staff Fellow	LDS, NIDDK
	T.L. Fong	Medical Staff Fellow	LDS, NIDDK

## COOPERATING UNITS (If any)

Georgetown University, Washington, D.C. (J. Gerin)

Walter Reed Army Institute of Research, Washington, D.C. (M. Sjogren)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Liver Diseases Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

3

## PROFESSIONAL

2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials of antiviral and/or immunomodulatory agents. Efforts are now being directed towards improving the therapeutic response rate to alpha interferon alone. Two studies using alpha interferon as therapy for chronic type B hepatitis are underway. The first is a randomized, controlled trial to reevaluate the effects of alpha interferon alone (at a dose of 10 million units three times weekly) compared to no therapy. Forty four patients have been entered into this study and 11 have now been treated with interferon and completed follow up (total 6 mos.). Among these patients, 5 (45%) cleared HBeAg from serum, 2 had a partial response with loss of DNA polymerase activity from serum but no clearance of HBeAg and the remaining 4 patients showed only a temporary partial inhibition of DNA polymerase activity in serum. The disease in controls did not improve. A second study is designed for patients who have not responded to interferon alone in previous studies. In these cases, the effect of pretreatment with a 4 week course of prednisone before administration of interferon is being evaluated. It is hoped that the immunostimulatory effects of rapid withdrawal of corticosteroids, by inducing an exacerbation of hepatitis activity will tend to optimize the antiviral effects of alpha interferon. Nine patients have been entered into this study so far, and all have completed the treatment regimen. Only one patient has responded with clearance of HBeAg and HBsAg from serum.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK, 53510-11 DDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Non-A, Non-B (Type C) Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A.M. DiBisceglie	Visiting Scientist	LDS, NIDDK
	J. Korenman	Medical Staff Fellow	LDS, NIDDK
	N.V. Bergasa	Medical Staff Fellow	LDS, NIDDK
	B.L. Baker	Medical Staff Fellow	LDS, NIDDK
	T.L. Fong	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

NIH Blood Bank (H.J. Alter, J.K. Shih), Armed Forces Institute of Pathology, Washington, D.C. (Z. Goodman), Chiron Corporation, Emeryville (M. Houghton, G. Kuo), Laboratory of Infectious Diseases, NIAID (S.M. Feinstone, R.H. Purcell).

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

2

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Patients with well-documented chronic non-A, non-B (NANB) hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients are available to evaluate experimental therapies for this disease. A prospective, randomized, placebo-controlled, double-blind trial of a six month course of human alpha interferon in patients with chronic NANB hepatitis has been completed. Forty one patients have completed the first 6 month (blinded) phase of the study. Twenty one received interferon and 20 placebo. Mean serum aminotransferase activities and liver histology improved significantly in interferon-treated patients but not in placebo recipients. Eighteen patients who initially received placebo were crossed over to receive interferon for 12 months. They have had a similar response to the patients treated for 6 months, although their long-term response rate may be higher.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53511-11 DDB

## PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trials of Therapies for Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	N.V. Bergasa	Medical Staff Fellow	LDS, NIDDK
	T.L. Fong	Medical Staff Fellow	LDS, NIDDK
	A. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	M.G. Swain	Guest Researcher	LDS, NIDDK
	M. Fried	Medical Staff Fellow	LDS, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Liver Diseases Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.50

## PROFESSIONAL:

.50

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative, destruction of small intrahepatic bile ducts. Because some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with symptomatic PBC. In a randomized controlled trial of chlorambucil therapy, treatment with the drug was associated with a decrease in the rate of increase of serum bilirubin, normalization of elevated serum IgM levels and an improvement in inflammatory cell infiltration in liver biopsies. These findings have prompted a search for safer and more effective immunosuppressive drugs with less carcinogenic potential than chlorambucol. Ten patients with PBC have been entered into an open trial of methotrexate therapy and are tolerating the drug well.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53514-07 DDB-

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunological Studies in Chronic Viral Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.A. Jones

Others: J.H. Hoofnagle

A. Di Bisceglie

R. Moreno-Otero

Chief

Director, DDDN

Visiting Scientist

Guest Researcher

LDS, NIDDK

NIDDK

LDS, NIDDK

LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Immunoregulation, NIAID (Dr. Julian Ambrus)  
Georgetown University (Dr. Tom Cupps)

LABORATORY BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK, 53515-04 DDB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Duck Hepatitis B Virus Infection

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A. M. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	J. Korenmann	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

Comparative Animal Unit (H. Robcis)  
Hepatitis Virus Section, NIAID (R. Miller)  
Clinical Oncology Program, NCI (H. Mitsuya, S. Broder)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

1.75

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

There are many similarities in structure and properties between the human hepatitis B virus (HBV) and the duck hepatitis B virus (DHBV). These similarities suggest that DHBV infection in ducks may be a useful experimental model of human HBV infection, particularly as HBV cannot be grown readily in cell culture. Ducks infected with DHBV at birth become chronic carriers of the virus, although they may not develop overt hepatitis. Some DHBV-infected ducks have been reported to develop hepatocellular carcinoma. The analogous human tumor is strongly linked etiologically with chronic hepatitis B infection. As a screening test for new effective therapies for chronic type B hepatitis in man, new antiviral and immunomodulatory agents are being assessed for their ability to suppress DHBV replication in ducks. Both 2',3'- dideoxyinosine and 2',3'-dideoxyguanosine have been shown to be potent inhibitors DHBV replication and to produce few side effects in ducks.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK, 53516-01 DDB

PERIOD COVERED: October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Studies of the Opiate System in Cholestatic Liver Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI	E. A. Jones	Chief	LDS, NIDDK
Others:	N. V. Bergasa	Medical Staff Fellow	LDS, NIDDK
	J. Vergalla	Medical Staff Fellow	LDS, NIDDK
	M.G. Swain	Medical Staff Fellow	LDS, NIDDK
	M. Fried	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH  
Digestive Diseases Branch

SECTION  
Liver Diseases Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.5	PROFESSIONAL 1	OTHER: .5
-------------------------	-------------------	--------------

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

A new system for continuously quantitating scratching activity has been devised. This system permits objective quantitative studies of the effectiveness of therapies for the pruritus of cholestasis to be undertaken. Using this system naloxone infusions have been shown to decrease scratching activity by about 50% in patients with pruritus due to primary biliary cirrhosis, suggesting that the opiate system may be implicated in the mediation of the pruritus of cholestasis. Rats with acute cholestasis due to bile duct ligation have been shown to have a mild analgesic state as evaluated with the tail flick assay. This analgesia can be reversed by naloxone. These findings are consistent with the syndrome of cholestasis being associated with increased opiate tone and raise the possibility that certain complications of cholestasis, such as pruritus, may be mediated by central opiate receptors and alleviated by the administration of opiate receptor antagonists that are effective when given orally (e.g. nalmefene).

ANNUAL REPORT OF THE  
MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The MCNEB continues basic and clinical investigations in the areas of molecular regulation and neuroendocrinology (Molecular Regulation and Neuroendocrinology Section, Bruce D. Weintraub, Chief); experimental diabetes, metabolism and nutrition (Experimental Diabetes, Metabolism and Nutrition Section, Samuel W. Cushman, Chief); and growth and development (Growth and Development Section, Matthew M. Rechler, Chief). The Branch has had many visiting fellows and associates, as well as international collaborations with the University of Milan, Italy; University of Marseilles, France; Karolinska Institute, Sweden; Postgraduate School of Obstetrics and Gynecology, University of Auckland, New Zealand; University of Naples, Italy; Department of Medicine, University of Gothenburg, Sweden; Endocrine Institute, Rambam Medical Center, Haifa, Israel; Department of Biochemistry, the University of Newcastle upon Tyne, England.

Dr. Weintraub was honored as a Visiting Professor at the Peter Bent Brigham Hospital, Brown University Hospital, Southwestern Medical Center and the Mayo Clinic. He was also chosen as a plenary speaker at the Asean Endocrine Society Meeting in Singapore, and the Australian Endocrine Society Meeting in Perth.

I THYROTROPIN, THYROTROPIN-RELEASING HORMONE AND THYROID HORMONE: MOLECULAR BIOLOGY, REGULATION, ACTION, AND PATHOPHYSIOLOGY.

A. Alterations in the carbohydrate structure of TSH in hypothyroidism

The oligosaccharide structure of secreted TSH in perinatal and mature rats with congenital-primary hypothyroidism was examined. Rat pituitaries from euthyroid control animals and those rendered hypothyroid by methimazole treatment were incubated with [ $^3\text{H}$ ]glucosamine in vitro. Secreted TSH was purified and oligosaccharides were enzymatically released and characterized by anion-exchange HPLC. In perinatal hypothyroid compared to control animals, oligosaccharides from TSH- $\alpha$  and TSH- $\beta$  contained more species with three or more negative charges. Moreover, perinatal hypothyroid animals demonstrated a dramatic increase in the ratio of sialylated to sulfated species within oligosaccharides of the same negative charge (2.9- to 7.4-fold increase for TSH- $\alpha$ ; 15.1- to 25.5-fold for TSH- $\beta$ ). In mature hypothyroid nine-week-old compared to control animals, changes were less pronounced, suggesting that endocrine regulation of oligosaccharide structure is dependent upon the maturational state of the animal. These changes were specific for TSH since glycosylation of free  $\alpha$  subunit (synthesized by the thyrotroph and gonadotroph) and of total glycoproteins was minimally altered by hypothyroidism. Together, these data provide direct evidence and characterization of specific changes in the structure of a secreted pituitary glycoprotein hormone occurring as a result of in vivo endocrine alterations during early development. Moreover, they provide a potential structural basis to explain the delayed clearance of both TSH and the gonadotropins with end-organ deficiency, which may have important implications for the in vivo biological activities of these hormones.

N. R. Thotakura, R. K. Desai, L. G. Bates, B. D. Weintraub



## B. Studies on the role of carbohydrate structure using glycosylation processing inhibitors

We examined the effect of various inhibitors of oligosaccharide processing on the content and secretion of newly synthesized TSH from dispersed hypothyroid rodent pituitary cells. 1-Deoxynojirimycin and N-methyl-1-deoxynojirimycin, both inhibitors of glucosidases I and II, decreased intracellular TSH (to 60-76% of control) and secreted TSH (to 60-63% of control) after a 1-hour incubation (pulse) with [35S]methionine and an 8-hour incubation (chase) in isotope-free media. In contrast, deoxymannojirimycin and swainsonine, inhibitors of mannosidase I and II, respectively, increased both intracellular TSH (to 267-309% of control) and secreted TSH (to 192% of control) at 8 hours. TSH oligosaccharides synthesized in the presence of these glucosidases and mannosidase inhibitors were largely sensitive to endo- $\beta$ -N-acetylglucosaminidase H (endo H), confirming inhibition of processing. Despite differences in oligosaccharide structure, the *in vitro* bioactivities of these secreted TSH isoforms were nearly identical. These data confirm and extend previous work performed with 1-deoxynojirimycin suggesting that glucosylated high mannose forms of TSH are more susceptible to intracellular degradation. The novel finding that deoxymannojirimycin and swainsonine increase secreted and total TSH above control levels suggests that non-glucosylated high mannose forms as well as hybrid-type oligosaccharides may facilitate secretion and direct TSH away from a natural degradation pathway.

Several stable mutant CHO cell lines producing hTSH molecules with altered carbohydrate chains are being established to extend the above studies.

.... B. S. Stannard, N. R. Thotakura, B. D. Weintraub.

## C. Structure-function relation studies of recombinant hTSH

Since the carbohydrate structure of a protein reflects the glycosylation apparatus of the host cells in which the protein is expressed, we examined the biological activity and metabolic clearance of recombinant human (rh) TSH expressed in Chinese hamster ovary (CHO) cells. RhTSH was produced by co-transfection of a cDNA for human choriogonadotropin  $\alpha$  and a hTSH  $\beta$  minigene into CHO cells, and stable transfectants with high rates of rhTSH production were selected. Carbohydrate compositional analysis of recombinant thyrotropin showed it to be highly sialylated with no GalNAc and therefore the absence of terminal sulfate moieties, which are both normally present in pituitary-derived TSH. Thus, this is a novel glycoform of hTSH containing only sialic acid-terminating oligosaccharides and is closer to hypothyroid hTSH. Immunologic activity of rhTSH was about two-fold lower and receptor-binding activity was similar to that of the standard pituitary TSH. Removal of sialic acid did not alter the immunologic and receptor-binding activities of rhTSH. The rhTSH was fully active in two different *in vitro* bioassays. However, rhTSH was about three-fold and eight-fold less potent in adenylyl cyclase stimulation in bovine thyroid membranes and cAMP production in FRTL-5 cells respectively than pituitary TSH. The lower *in vitro* biological activity may be due to its highly sialylated oligosaccharide chains. The lower potency in the above assays was reversed by sialic acid removal from rhTSH and the asialo-rhTSH showed potency similar to pituitary hTSH in the above two assays. The rhTSH had a 2.6-fold lower metabolic clearance rate than pituitary TSH, due to a decrease in the rapid as well as slow phases of clearance. After sialic acid removal, the rhTSH was cleared much faster than pituitary hTSH, showing that its longer plasma half-life was due to its higher sialylation. We have for the first time studied the biological properties of rhTSH produced in large scale and compared it with those of pituitary hTSH. It is also possible for the first time to study the role of terminal as well as individual sugar residues of hTSH and assess the role of sialic acid vs. sulfate in hTSH.

Several stable CHO cell lines producing mutant hTSH molecules with deleted carbohydrate chains are being established. Now it will be possible to study the assembly, secretion and biological function of the individual glycosylation sites in hTSH.

... N. R. Thotakura, R. K. Desai, L. G. Bates, B. D. Weintraub

#### D. Structure-function studies on TSH using site-directed mutagenesis

In order to study structure-function relationships of human thyrotropin, a human embryonal kidney cell line (293 cells) was transfected with an  $\alpha$  subunit cDNA and a TSH- $\beta$  subunit minigene. Site-directed mutagenesis was used to alter the TSH- $\beta$  subunit and produce mutant thyrotropins. The glycosylation recognition sequence Asn-X-Ser/Thr extends from amino acids 23 to 25 of the TSH- $\beta$  chain. The effects of glycosylation on the secretion of TSH was studied by changing amino acids 23 and 25 to Gln (Gln 23) and Tyr (Tyr 25) respectively, using the techniques of site-directed mutagenesis. A change in amino acid 25 to Ser (Ser 25), on the other hand, would retain glycosylation. The predicted changes in glycosylation were confirmed by the inability of Gln 23 and Tyr 25 mutants to incorporate  $^3\text{H}$ -oligosaccharides. The marked reduction in secretion (greater than 90%) of the Gln 23 and Tyr 25 mutants demonstrates the importance of glycosylation of TSH in this process. We will be able to study the mechanism for this decrease in secretion by performing pulse-chase studies in stably transformed TSH secreting cell lines, to be described below.

Despite the fact that glycosylation of the Ser 25 mutant was retained, secretion was only 30% of that of wild type. The probable reason for this is the proximity of this site to the CAGYC region (amino acids 27-31), an area of the  $\beta$  chain thought to be important for subunit combination. A mutation in the CAGYC region itself (Arg 29) resulted in the absence of detectable TSH or TSH $\beta$  subunit. Decreased secretion of any of the mutants described was not due to differences in gene transcription or an increase in intracellular accumulation of TSH or  $\beta$  subunit. We also engineered a carboxyterminal mutant (Stop 112), shortening the TSH $\beta$  subunit from the 118 amino acids predicted by the gene sequence to the peptide's observed length of 112 amino acids. Production of this TSH mutant was significantly higher than that of the wild-type. A possible explanation for this finding is that shortening of the TSH- $\beta$  subunit from 118 to 112 amino acids enhances  $\beta$  subunit stabilization and subsequent dimerization.

... R. W. Lash, R. K. Desai, N. R. Thotakura, B. D. Weintraub

#### E. Generation of stable cell lines producing TSH or TSH mutants

The CGA cDNA and the minigene of TSH $\beta$  (or the TSH $\beta$  mutants described above) were cut out of the Hind III site of pAV<sub>2</sub> and inserted into the Sal I site of pMAM<sub>neo</sub>. This expression vector has an RSV-LTR enhancer linked to the dexamethasone inducible MMTV-LTR promoter, together with the gene encoding for the production of aminoglycoside phosphotransferase. Chinese hamster ovary (CHO) cells were co-transfected with the pMAM<sub>neo</sub>- $\alpha$  and pMAM<sub>neo</sub>- $\beta$  (or pMAM<sub>neo</sub>- $\beta$  mutant) vectors. Cells were then grown in the presence of G418 (an analogue of neomycin) and surviving colonies were screened for TSH production. In the presence of dexamethasone, the Stop 112 mutant was secreted into the media at a concentration of 150 IU/l. Bioactivity of wild and mutant TSH will soon be tested using FRTL-5 cells, and pulse-chase studies in the stable cell lines will elucidate the mechanisms for changes in secretion levels of the mutant TSH molecules. Furthermore, growth of the mutant cell lines in bioreactors will allow isolation of sufficient quantities of material to further characterize biological properties (metabolic clearance rate, in vivo targeting, receptor binding) of the various mutant TSH molecules.

F. Mutations of the Beta Thyroid Hormone Receptor Gene in Generalized Thyroid Hormone Resistance Causing Aberrant TSH- $\beta$  Regulation.

Generalized thyroid hormone resistance (GTHR) is a disorder of thyroid hormone action characterized by elevated free thyroid hormones and TSH, and inappropriate clinical and biochemical signs of euthyroidism or hypothyroidism. It is usually transmitted as a familial syndrome with an autosomal dominant pattern of inheritance. We have previously shown that in a kindred, A, the gene for GTHR is tightly linked to one of the two known thyroid hormone receptor genes, c-erbA $\beta$ , on chromosome 3. To study the role of the thyroid hormone receptor gene in this syndrome, we have conducted further linkage analyses in two other kindreds, B and D, with differing degrees of thyroid hormone insensitivity. There was also linkage between c-erbA $\beta$  and GTHR loci in these two kindreds, and the combined maximum lod score for all three kindreds at a recombination fraction of 0 was 5.77. We investigated the defect in c-erbA $\beta$  in Kindred A by sequencing the T3-binding domain in the 3'-region of fibroblast c-erbA $\beta$  cDNA and leukocyte c-erbA $\beta$  genomic DNA. A base substitution, cytosine to adenine, was found at cDNA position 1643 which altered the proline codon to a histidine codon. This base substitution was only found in one allele of affected members of Kindred A, as expected for a dominant disease, and was not found in unaffected members, in two other Kindreds, or in 92 random c-erbA $\beta$  alleles. Because of this absolute linkage with the abnormal phenotype, and the fact that the mutation is predicted to alter the secondary structure of the crucial T3-binding domain of the c-erbA $\beta$  receptor it was likely to represent a c-erbA $\beta$  mutation responsible for GTHR in kindred A.

The functional significance of the mutation in the A Kindred was demonstrated by transcribing mutant receptor cDNA and translating the messenger RNA in a reticulocyte lysate system. Compared to normal receptor, the mutant receptor bound T3 with 10-fold less affinity, although it bound to the thyroid hormone inhibitory element in the TSH- $\beta$  gene with equal affinity. These data suggest that decreased binding of T3 prevents a conformational change in the mutant receptor necessary for transcriptional activity, despite normal DNA binding. Moreover, the mutant receptor appears to form a heterodimer with the normal receptor derived from the nonmutant allele and causes a dominant negative effect on TSH- $\beta$  transcription.

Recently we have discovered novel and completely different point mutations of the thyroid hormone  $\beta$  receptor in ten other kindreds. We are currently employing new DNA mismatch and chemical cleavage techniques to screen rapidly for mutations in 15 other families that we have studied previously at NIH. Characterization of the functional properties of various mutant receptor genes should provide novel insights into the mechanisms of thyroid hormone action in man.

... S. J. Usala, A. J. Mixson, R. Parrilla, B. D. Weintraub

G. Characterization of the thyroid hormone inhibitory element in the human thyrotropin  $\beta$  subunit gene.

The first exon of the hTSH $\beta$  gene has been demonstrated in our laboratory to contain a major thyroid hormone inhibitory element. In order to provide a detailed characterization of this element, we performed a scanning mutation analysis by transient transfection of hTSH $\beta$  expression vectors containing five bp cassette mutations within the first exon of the hTSH $\beta$  gene, in conjunction with avidin-biotin complex DNA binding and DNase I footprinting. Various -1200 to +37 bp fragments of the hTSH $\beta$  gene containing consecutive five deoxythymidine substitution mutations (+3 to +7, +13 to +17, +18 to +22, +23 to +27, +28 to +32, and +33 to +37) were placed within a luciferase reporter plasmid and transiently transfected into human

embryonal kidney cells using the calcium phosphate precipitate method. A thymidine kinase promoter-growth hormone reporter plasmid was cotransfected as an internal control. The cells were treated with  $10^{-8}$  M  $T_3$  for 24 hours and relative light units of luciferase activity measured. Wild type constructs were inhibited 38% ( $\pm 3.5\%$ ) by  $T_3$  as compared to control. Mutations +33 to +37 and +28 to +32 lost  $T_3$  inhibition to 13% ( $\pm 5.7$ ) and 3% ( $\pm 2.1$ ), respectively. Inhibition returned to control levels in mutation +23 to +27 but was again reduced to 9.5% ( $\pm 4.1$ ) in +18 to +22. Further 5' mutations +13 to +17 and +3 to +7 showed almost total loss of inhibition with 1.2% ( $\pm 1.1$ ) and 2.4% ( $\pm 1.6$ ), respectively. Human  $^{35}\text{S}$ -labeled c-erbA- $\beta$  protein was synthesized in an *in vitro* transcription-translation system and utilized for DNase footprinting and DNA binding assay for the first exon of the hTSH $\beta$  gene. A radiolabeled hTSH $\beta$  DNA fragment (-199 to +79) was exposed to c-erbA- $\beta$  protein and unprogrammed cell lysate as control. A strong footprint was noted from +3 to +16 and a weaker footprint from +33 to +35. Multiple fragments in the DNA binding assay localized binding to two sites, a high avidity site extending from +1 to +15 and a lower avidity site from +3- to +44. In conclusion, functional and binding studies indicate at least two sites of interaction between the thyroid hormone receptor and the first exon of the hTSH $\beta$  gene; the upstream site exhibits stronger avidity for c-erbA- $\beta$  and functionally extends over a longer distance. These results suggest that thyroid hormone receptors, binding to at least two sites in the first exon, act in conjunction to mediate  $T_3$  inhibition of hTSH $\beta$  expression.

... D. L. Bodenner, F. E. Wondisford, J. H. McClaskey, B. D. Weintraub

#### H. Cloning and regulation of the human prepro-TRH gene (In collaboration with Dr. John Wilber, University of Maryland Medical Center)

We have characterized genomic DNA encoding human prepro-TRH, containing six repetitive coding sequences for TRH. Toward elucidation of TRH gene regulation by L- $T_3$ , we have developed plasmid chimeric constructs containing the 5' flanking region of ppTRH fused to the luciferase reporter in the plasmid pVSO. Evidence from our laboratory and others had indicated that thyroid hormones inhibit prepro-TRH mRNA transcription, and sequences homologous to the thyroid hormone inhibitory elements in rat and human TSH  $\beta$ -genes have been identified in ppTRH. 5' deletion mutants (-900 to +54, -600 to +54, -250 to +54) were transfected into rat GH $_3$  cells in the presence or absence of L- $T_3$  ( $10^{-11}$  to  $10^{-8}$  M) by the calcium phosphate method. In the full length mutant, substantial TRH gene promoter activity was present (580 light units/mcg protein). Deletion mutants showed progressively reduced promoter activity, to 40% and 10% respectively of the activity of the full length chimeric construct (-900 +65 bp). Importantly, the addition of L- $T_3$  resulted in progressive inhibition of gene expression with maximal inhibition (-71%) at  $10^{-9}$  M L- $T_3$  ( $p < 0.001$ ). In contrast, only slight and not significant inhibition (-13%) by L- $T_3$  at  $10^{-9}$  was seen in pSV40 TRH enhancerless constructs. Conclusions: (1) The human prepro-TRH 5' flanking region promoter in pSVO plasmid constructs is strongly expressed in GH $_3$  cells, (2) there are at least two enhancer elements between bp -900 and +54 of the ppTRH gene, (3) L- $T_3$  can inhibit TRH human gene expression directly in GH $_3$  cells.

... S. Radovick, F. E. Wondisford, B. D. Weintraub

#### I. Cloning and characterization of the human gonadotropin-releasing hormone gene.

We have cloned and characterized the human gonadotropin-releasing hormone (GnRH) gene. The expression of the 5' flanking region and downstream elements of the GnRH gene was examined in a human choriocarcinoma cell line, JEG-3. Transient expression studies were performed with a series of chimeric expression vectors containing -1130 to +5 bp (promoter region), -1130 to +6.1 bp (through the first exon), -1130 to 1073 bp (through the second exon and -1130 to +2684 bp (through the third exon) fused to the firefly luciferase gene (LUC). All

expression vectors were constructed to preserve the normal splicing patterns in the human GnRH gene. The -1130/+5 bp construct showed very low basal activity and the addition of the first exon (-1130/+61 bp construct) did not significantly affect activity. The -1130/+1073 bp construct and the -1130/+2684 bp construct revealed a 3.8-fold and 6.0-fold increase in activity compared to the -1130/+5 bp construct. These data indicate that region(s) downstream from the transcriptional start site of the human GnRH gene is important in enhancing basal expression.

Although we fused either the first intron or the second intron upstream of the heterologous thymidine kinase (TK) promoter in pTKLUC, neither the first nor the second intron could enhance activity of the TK promoter. The first intron was also fused in both orientations downstream of the luciferase gene in the -1130/+5 bp LUC construct. The first intron did not enhance activity of the homologous promoter. These data suggest that DNA sequences downstream from the transcriptional start site of the human GnRH gene may enhance expression in a promoter specific and orientation and position-dependent manner.

.... Y. Nakayama, S. Radovick, F. E. Wondisford, B. D. Weintraub

#### J. Molecular basis of familial precocious puberty and hypogonadotropic hypogonadism.

Familial central precocious puberty (CPP) is due to an early activation of the hypothalamic-pituitary-gonadal axis, while idiopathic hypogonadotropic hypogonadism (IHH) results from lack of hypothalamic GnRH secretion. Although familial cases of both disorders have been recognized, the modes of inheritance have not been fully elucidated. We have examined the structure of the GnRH gene in a family with familial central precocious puberty (CPP) and a family with idiopathic hypogonadotropic hypogonadism (IHH) using Southern blot analysis and sequencing of cloned polymerase chain reaction product. Southern blot analyses using the human placental GnRH cDNA probe revealed the same size bands as those found by this analysis in normal individuals. Sequencing of exon 2 of the GnRH gene from these families, including the exon-intron borders, revealed a polymorphism in the signal sequence of GnRH that predicts an amino acid change from tryptophan (nucleotide sequence:TGG) to serine (TCG) at the -8 position of the GnRH preprohormone. Although this polymorphism did not cosegregate with the clinical disorder in either family, this novel polymorphism may prove useful in the evaluation of linkage to the GnRH gene in other families with this disorder. No other nucleotide sequence abnormality was found in 1.2 kb of the 5' flanking region of the four exons and their splice sites.

.... Y. Nakayama, S. Radovick, F. E. Wondisford, B. D. Weintraub.

#### II. Insulin-like growth factors

We have continued our studies of the insulin-like growth factors (IGFs), their receptors and binding proteins to understand the physiological and pathological role of the IGFs. During the past year we have demonstrated that: (1) adult rat serum contains a growth hormone dependent, glycosylated IGF-binding protein (IGFBP-3), and nonglycosylated binding proteins of 30 and 24 kDa; (2) the main binding protein in fetal rat serum is IGFBP-2, which is not present in adult rat serum; (3) and IGFBP-2 mRNA is expressed in all fetal rat tissues, with highest levels in liver, followed by brain and stomach. In adult rats, IGFBP-2 mRNA levels are lower in liver and other tissues, but not in brain; (4) IGFBP-1 mRNA also is predominantly expressed in fetal liver, and is decreased in adult liver. Levels in fetal kidney and brain are significantly lower than IGFBP-2 mRNA; (5) IGFBP-2 mRNA is localized to the choroid plexus in adult rat brain, and the IGFBP-2 is the major IGF binding protein in rat cerebrospinal fluid; (6) In the brain of midgestational rat embryos, IGFBP-2 mRNA is expressed in the choroid plexus epithelium, the progenitor of the

posterior pituitary, and the floor plate. IGF-II mRNA is expressed in some adjacent cells (the choroid mesenchyme and progenitors of the anterior and intermediate lobes of the pituitary), but not in the floor plate; (7) IGFBP-2 was identified in a human rhabdomyosarcoma cell line and in human cerebrospinal fluid; (8) In adult rat liver, IGFBP-2 mRNA is increased 10-fold after hypophysectomy, fasting, or streptozotocin diabetes; IGFBP-1 mRNA is not increased after hypophysectomy, is increased after prolonged fasting, and is increased 100-fold in diabetes; (9) In rat hepatoma cell line, dexamethasone increases IGFBP-1 gene transcription; (10) IGF-II is an autocrine growth factor for a human neuroblastoma cell line, and acts via the IGF-I receptor; (11) IGF-I stimulates motility of a human melanoma cell line via the IGF-I receptor.

.... M. M. Rechler, A. L. Brown, D. E. Graham, C. C. Orlowski, Y. W.-H. Yang,  
J. A. Romanus, L. Tseng, G. T. Ooi, D. S. Straus

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55000-18 MCNE

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

## Biosynthesis, Glycosylation, and Action of Thyrotropin

PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. D. Weintraub	Chief	MCNEB, NIDDK
OTHERS:	N. R. Thotakura	Visiting Associate	MCNEB, NIDDK
	R. K. Desai	Visiting Fellow	MCNEB, NIDDK
	L. G. Bates	Biologist	MCNEB, NIDDK
	R. W. Lash	Senior Staff Fellow	MCNEB, NIDDK
	L. Joshi	Senior Staff Fellow	MCNEB, NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Molecular Regulation and Neuroendocrinology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
 ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The oligosaccharide structure of secreted TSH in perinatal and mature rats with hypothyroidism was compared to euthyroid controls using newly developed methods of anion exchange HPLC. Compared to control TSH, hypothyroid TSH showed relatively more sialic acid and less terminal sulfate, as well as more complicated structure with 3 or more negative charges, probably representing multiantennary structures. TSH derived from chronically hypothyroid animals was similar to recombinant TSH produced after transfection of  $\alpha$  and TSH- $\beta$  genes into Chinese hamster ovary cells. Such recombinant TSH was completely sialylated and showed no evidence of GalNAc or terminal sulfate moieties. The recombinant hormone was 3- to 8-fold less potent in various *in vitro* bioassays, but showed a considerably longer half-life when injected into euthyroid rats. Hypothyroidism as well as recombinant TSH will be useful in determining the physiologic roles of sialic acid vs. sulfate in the action of human TSH. In addition, the recombinant hormone will be of clinical utility in the diagnosis and treatment of patients with thyroid cancer.

Using site-directed mutagenesis, we are currently determining the structure-function relationships of human TSH. We have already mutated various amino acids as well as glycosylation sites and determined important functional roles of various codons in the  $\beta$  subunit. We are also generating several stable cell lines producing these TSH mutants so that detailed studies of biosynthesis, degradation and secretion can be determined. Finally, we have used various inhibitors of oligosaccharide processing to determine the role of carbohydrate structure on the secretion of TSH from dispersed rodent pituitary cells. Inhibitors of both glucosidases as well as mannosidases impair the assembly and secretion of TSH and the high mannose forms containing glucose are particularly susceptible to intracellular degradation. Several stable mutant cell lines producing TSH molecules with altered carbohydrate chains are being established to extend these studies.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 DK 55001-14 MC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Regulation and Action of Thyrotropin

## PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. D. Weintraub Chief MCNEB, NIDDK

OTHERS: N. R. Thotakura Visiting Associate MCNEB, NIDDK  
N. Gesundheit Senior Staff Fellow MCNEB, NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Molecular Regulation and Neuroendocrinology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been cancelled.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55002-10 MCNEB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Molecular Biology of Pituitary Glycoprotein Hormones and Hypothalamic Releasing Hormones

PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B.D. Weintraub	Chief	MCNEB, NIDDK
Others:	F.E. Wondisford	Senior Staff Fellow	MCNEB, NIDDK
	J. McClaskey	Senior Staff Fellow	MCNEB, NIDDK
	D. L. Bodenner	Medical Staff Fellow	MCNEB, NIDDK
	Y. Nakayama	Visiting Fellow	MCNEB, NIDDK
	S. Radovick	Senior Staff Fellow	MCNEB, NIDDK

## COOPERATING UNITS (if any)

Dr. John F. Wilber, University of Maryland Medical Center, Baltimore, MD

LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch

SECTION Molecular Regulation and Neuroendocrinology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	5.3	PROFESSIONAL:	5.3	OTHER:	0
------------------	-----	---------------	-----	--------	---

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither

☐ (a1) Minors
 ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have cloned the genes for several human pituitary glycoprotein hormones, as well as hypothalamic releasing hormones, including the  $\beta$  subunit of human thyrotropin, as well as human prepro-TRH and human gonadotropin-releasing hormone. For the human thyrotropin  $\beta$  subunit gene we have localized a major thyroid hormone inhibitory element in the first untranslated exon of this gene using a combination of scanning mutational analysis as well as DNA, DNase I footprinting in conjunction with avidin-biotin complex DNA binding. This inhibitory element appeared to be comprised of two distinct half-sites, one located at the 5' end of the exon and another toward the 3' end of the exon. These various results suggest that thyroid hormone receptors binding to at least two sites in the first exon act in conjunction to mediate T3 inhibition of TSH- $\beta$  expression. We are currently trying to localize the regions responsible for T3 inhibition of human prepro-TRH gene expression, which seems to be located in the 5' flanking region from -900 to +54. The coding regions of human prepro-TRH and gonadotropin-releasing hormone genes have been completely elucidated. The former hypothalamic hormone gene was found to have 6 repetitive coding sequences for TRH which differs from the number in lower species. The human gonadotropin-releasing hormone gene was found to contain DNA sequences downstream from the transcriptional start site that enhanced expression in a promoter-specific and position-dependent manner. Finally, using a novel polymorphism in the DNA sequence of the gonadotropin-releasing hormone gene, we have shown in a family familial central precocious puberty, as well as in another family with idiopathic hypogonadotropic hypogonadism that these conditions were not related to a fundamental abnormality in this gene. This polymorphism may prove useful in the evaluation of linkage to the GnRH gene in other families with this disorder. These studies will also draw attention to other mechanisms for abnormal pubertal function in man not directly associated with the GnRH gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55006-17 MCNE

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Insulin-like Growth Factors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	M.M. Rechler	Chief, GD Section	MCNEB, NIDDK
Others:	A.L. Brown	Staff Fellow	MCNEB, NIDDK
	D.E. Graham	Microbiologist	MCNEB, NIDDK
	C.C. Orłowski	Staff Fellow	MCNEB, NIDDK
	Y.W.-H. Yang	Staff Fellow	MCNEB, NIDDK
	J.A. Romanus	Chemist	MCNEB, NIDDK
	L.Tseng	Chemist	MCNEB, NIDDK
	G.T. Ooi	Visiting Fellow	MCNEB, NIDDK
	D.S. Straus	Special Volunteer	MCNEB, NIDDK

COOPERATING UNITS (if any) MB NCI (S.P. Nissley); DCBDLP NCI (M.L. Stracke, E. Schiffman); PB NCI (O.M. El-Badry, M.A. Israel); Univ. of Naples, Italy (C.B. Bruni, L. Chiariotti); Columbia University (T.L. Wood, J.E. Pintar)

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Growth and Development Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

7.25

## PROFESSIONAL:

6.25

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have continued our studies of the insulin-like growth factors (IGFs), their receptors and binding proteins to understand the physiological and pathological role of the IGFs. During the past year we have demonstrated that: (1) adult rat serum contains a growth hormone dependent, glycosylated IGF-binding protein (IGFBP-3), and nonglycosylated binding proteins of 30 and 24 kDa; (2) the main binding protein in fetal rat serum is IGFBP-2, which is not present in adult rat serum; (3) IGFBP-2 mRNA is expressed in all fetal rat tissues, with highest levels in liver, followed by brain and stomach. In adult rats, IGFBP-2 mRNA levels are lower in liver and other tissues, but not in brain; (4) IGFBP-1 mRNA also is predominantly expressed in fetal liver, and is decreased in adult liver. Levels in fetal kidney and brain are significantly lower than IGFBP-2 mRNA; (5) IGFBP-2 mRNA is localized to the choroid plexus in adult rat brain, and IGFBP-2 is the major IGF binding protein in rat cerebrospinal fluid; (6) In the brain of midgestational rat embryos, IGFBP-2 mRNA is expressed in the choroid plexus epithelium, the progenitor of the posterior pituitary, and the floor plate. IGF-II mRNA is expressed in some adjacent cells (the choroid mesenchyme and progenitors of the anterior and intermediate lobes of the pituitary), but not in the floor plate; (7) IGFBP-2 was identified in a human rhabdomyosarcoma cell line and in human cerebrospinal fluid; (8) In adult rat liver, IGFBP-2 mRNA is increased 10-fold after hypophysectomy, fasting, or streptozotocin diabetes; IGFBP-1 mRNA is not increased after hypophysectomy, is increased after prolonged fasting, and is increased 100-fold in diabetes; (9) In a rat hepatoma cell line, dexamethasone increases IGFBP-1 gene transcription; (10) IGF-II is an autocrine growth factor for a human neuroblastoma cell line, and acts via the IGF-I receptor; (11) IGF-I stimulates motility of a human melanoma cell line via the IGF-I receptor.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55007-12 MCNE

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-cell interaction

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: T. M. Weber

Staff Fellow

MCNEB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project remains current but no summary was available

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 55008-12 MCNE

PERIOD COVERED

October 1, 1989 to September 30 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.W. Cushman

Chief, EDMNS

MCNEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project remains current but no summary was available.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55010-09 MCNE

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in insulin's action in insulin-dependent diabetes mellitus

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman

Chief, EDMNS

MCNEB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project remains current but no summary was available.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 55012-08 MCNE

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Hormone Binding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman

Chief, EDMNS

MCNEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project remains current but no summary was available.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55013-07 MCNE

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Counterregulation of Insulin's Action by Catecholamines

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: I. A. Simpson

Visiting Scientist

MCNEB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project remains current but no summary was available.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 55014-07 MCNE

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action with Fasting/Refeeding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman

Chief, EDMNS

MCNEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project remains current but no summary was available.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-55015-01 MCNE

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Mutations of the Thyroid Hormone Receptor Gene in Patients with Thyroid Hormone Resistance

## PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. D. Weintraub Chief MCNEB, NIDDK

OTHERS: A. J. Mixson Senior Staff Fellow MCNEB, NIDDK

R. Parrilla Special Volunteer MCNEB, NIDDK

P. Hauser Special Volunteer MCNEB, NIDDK

## COOPERATING UNITS (if any)

Dr. Stephen J. Usala, Section of Endocrinology, East Carolina School of Medicine, Greenville, NC

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Molecular Regulation and Neuroendocrinology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Generalized thyroid hormone resistance is a disorder of thyroid hormone action characterized by elevated free thyroid hormones and TSH, as well as inappropriate clinical and biochemical signs of euthyroidism or hypothyroidism. Using restriction fragment length polymorphism, we initially linked this disorder to one of the two known thyroid hormone receptor genes, c-erbA- $\beta$  on chromosome 3. Subsequently we have shown in ten families that this disease is caused by mutations in the ligand binding domain of the  $\beta$  receptor. In each of ten families characterized there have been unique defects all of which have resulted in nonconservative changes in amino acids that have altered the T3 binding properties of the receptor, while causing no apparent change in DNA binding properties. These data suggest that decreased binding of T3 prevents a conformational change in the mutant receptor necessary for transcriptional activity.

We are currently elucidating the molecular defects in an additional 15 families studied at NIH and are attempting to correlate the unique molecular defects with various clinical manifestations of this syndrome. We are also attempting to express high levels of the receptor using various expression systems. Such studies should allow for the first time an elucidation of the physiologic role of the  $\alpha$  and  $\beta$  thyroid hormone receptors for mediating thyroid hormone action in man.

ANNUAL REPORT OF THE LABORATORY OF STRUCTURAL BIOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. BIOLOGY OF COMPLEX CARBOHYDRATES

The carbohydrate-binding properties of the leech anticoagulant antistasin indicated that the protein binds with high affinity to the glycolipid sulfatide. Comparisons of the amino acid sequences of antistasin and other sulfatide or heparin-binding proteins revealed at 14 amino acid region of homology. For example, sequence homologies occur in thrombospondin, von Willebrand factor,  $\beta$ 2-glycoprotein I, and collagen Type IV, all of which bind heparin and/or sulfatide. Homologies were also found with coat proteins from malaria circumsporozoites and Herpes simplex I, and the alternate complement pathway protein, properdin.

The mannose binding protein has been purified to homogeneity. This protein binds with high affinity to Lc3Cer (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4 $\beta$ 1-lceramide) and nLc5Cer (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-4Gal $\beta$ 1-4Glc $\beta$ 1-lceramide) and can be used as a probe to determine the level of these substances in tissues. This technique was utilized to demonstrate that one sample of chronic myeloid leukemia cells contains both Lc3Cer and nLc5Cer.

....Drs. V. Ginsburg, G. Holt, N.H. Guo, L. Zhang, M. Kyogashima, and H. Krivan

II. EXPRESSION AND FUNCTION OF BACTERIAL CELL SURFACE COMPONENTS  
IN PATHOGENESIS

A bacterial pathogen must be able to invade and subsequently multiply, or at least survive in its host. Normally, the host defenses, such as serum-killing and phagocytosis, successfully oppose the invader and where an infection becomes established, administration of antibiotics may provide additional time for the host to eliminate invading pathogen. These studies focus on how the cell surface components of pathogenic bacteria function in resistance to host defense mechanisms and antibiotics.

A. Resistance to serum killing phagocytosis. Both capsular polysaccharide and O-antigen polysaccharide of lipopolysaccharide (LPS) have a role in the survival of the Salmonellae in the host. The specific structure of the LPS O-antigen is responsible for the rate and extent of complement activation which, in turn, is responsible for the serum-killing of the cell. The Vi-antigen of Salmonella typhi is a surface capsular polysaccharide that has long been postulated to provide protection against host defenses. Using isogenic pairs of Salmonellae strains differing only in the presence of absence of the Vi-antigen, we have shown that the Vi-antigen provides protection against phagocytosis but not serum-killing.

B. Escherichia coli and other Gram-negative bacteria grown in the presence of salicylate or aspirin become phenotypically resistant to a variety of commonly used antibiotics including cephalosporins, norfloxacin, and tetracycline. This resistance often extends beyond the levels usually achieved during antibiotic therapy is due, at least in part, to surface alterations that reduce the permeation of the antibiotic into the cell. These changes extend to bacteria grown in serum.

These observations may contra-indicate concomitant administration of aspirin and certain antibiotics.

..... Dr. J. Foulds

### III. METABOLISM AND ROLE OF POLYSACCHARIDE SULFATES

Fucoidan, a fucose containing sulfated carbohydrate polymer, inhibits a wide range of physiological processes which involve cell-cell interactions such as sperm-egg fertilization, tumor growth and the binding of lymphocytes to the endothelial cells. Some of these effects are exhibited by sulfated fucose. To evaluate the role of monomeric fucose sulfates, suitably blocked derivatives of methyl-L-fucoside have been prepared. From these, mono- and disulfated derivatives of known structure will be synthesized.

....Dr. I. Leder

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Biology of Complex Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Victor Ginsburg, Ph.D.	Chief, LSB	NIDDK
Others:	Gordon Holt, Ph.D.	Staff Fellow	LSB NIDDK
	Neng Hua Guo, M.D.	Visiting Fellow	LSB NIDDK
	Lijuan Zhang	Visiting Fellow	LSB NIDDK
	Mamoru Kyogashima, Ph.D., M.D.	Visiting Fellow	LSB NIDDK
	Howard Krivan, Ph.D.	Staff Fellow	LSB NIDDK

COOPERATING UNITS (if any)

James Mulshine, NCI

LAB/BRANCH Laboratory of Structural Biology

SECTION Section on Biochemistry

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

7

PROFESSIONAL:

6

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The carbohydrate-binding properties of the leech anticoagulant antistasin indicated that the protein binds with high affinity to the glycolipid sulfatide. Comparisons of the amino acid sequences of antistasin and other sulfatide or heparin-binding proteins revealed at 14 amino acid region of homology. For example, sequence homologies occur in thrombospondin, von Willebrand factor,  $\beta$ 2-glycoprotein I, and collagen Type IV, all of which bind heparin and/or sulfatide. Homologies were also found with coat proteins from malaria circumsporozoites and Herpes simplex I, and the alternate complement pathway protein, properdin.

The mannose binding protein has been purified to homogeneity. This protein binds with high affinity to Lc3Cer (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4 $\beta$ 1-lceramide) and nLc5Cer (GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-4Gal $\beta$ 1-4Glc $\beta$ 1-lceramide) and can be used as a probe to determine the level of these substances in tissues. This technique was utilized to demonstrate that one sample of chronic myeloid leukemia cells contains both Lc3Cer and nLc5Cer.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 57001-13 LSB
PERIOD COVERED October 1, 1989 to September 30, 1990		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Metabolism and Role of Polysaccharide Sulfates		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between; padding: 10px;"> <div>PI: Irwin G. Leder</div> <div>Research Chemist</div> <div>LSB NIDDK</div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Structural Biology		
SECTION Section on Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS <div style="text-align: center;">1</div>	PROFESSIONAL <div style="text-align: center;">1</div>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)  <p>Fucoidan, a fucose containing sulfated carbohydrate polymer, inhibits a wide range of physiological processes which involve cell-cell interactions such as sperm-egg fertilization, tumor growth and the binding of lymphocytes to the endothelial cells. Some of these effects are exhibited by sulfated fucose. To evaluate the role of monomeric fucose sulfates, suitably blocked derivatives of methyl-L-fucoside have been prepared. From these, mono- and disulfated derivatives of known structure will be synthesized.</p> <p>....Dr. I. Leder</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 57002-16 LSB

PERIOD COVERED  
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Expression and Function of Bacterial Cell Surface Components in Pathogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: John Foulds Research Biochemist LSB NIDDK

COOPERATING UNITS (if any)

Judeh Rosner, LMB, NIDDK

LAB/BRANCH

Laboratory of Structural Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A bacterial pathogen must be able to invade and subsequently multiply, or at least survive in its host. Normally, the host defenses, such as serum-killing and phagocytosis, successfully oppose the invader. Where an infection becomes established, administration of antibiotics may provide additional time for the host to marshall its defenses eliminate invading pathogen. These studies focus on how the cell surface components of pathogenic bacteria function in resistance to host defense mechanisms and antibiotics.

A) Resistance to serum killing phagocytosis. Both capsular polysaccharide and O-antigen polysaccharide of lipopolysaccharide (LPS) have a role in the survival of the Salmonellae in the host. The specific structure of the LPS O-antigen is responsible for the rate and extent of complement activation which, in turn, is responsible for the serum-killing of the cell. The Vi-antigen of Salmonella typhi is a surface capsular polysaccharide that has long been postulated to provide protection against host defenses. Using isogenic pairs of Salmonellae strains differing only in the presence of absence of the Vi-antigen, we have shown that the Vi-antigen provides protection against phagocytosis but not serum-killing.

B) Escherichia coli and other Gram-negative bacteria grown in the presence of salicylate or aspirin become phenotypically resistant to a variety of commonly used antibiotics including cephalosporins, norfloxacin, and tetracycline. This resistance often extends beyond the levels usually achieved during antibiotic therapy is due, at least in part, to surface alterations that reduce the permeation of the antibiotic into the cell. These changes extend to bacteria grown in serum. These observations may contra-indicate that the concomitant administration of aspirin and certain antibiotics

ANNUAL REPORT  
THE LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The LMCB comprises several diverse groups who nevertheless study a common theme of gene regulation in mammalian cells and their function in the pathophysiology of various disease states. One group, led by Dr. Carter, studies gene regulation in mammalian cell systems and is particularly interested in developing efficient vector systems for delivery of genes into cells. The second group, led by Dr. Oka, is generally interested in the endocrine control of differentiation of the mouse mammary gland and has focused on physiological effects of EGF and the molecular biology of various genes which are important in this process. A third project led by Dr. Tietze, is aimed at understanding the molecular basis of several human genetic defects which result in lysosomal storage diseases. This project is conducted in collaboration with workers in NICHD.

In the past year several changes have occurred in the organization of LMCB. Primarily, we have begun the process of transferring the Section on Steroid Hormones (led by S. Simons) to LMCB. Although formal administrative transfer is still in process for all operational purposes the Steroid Hormone Section already functions as part of LMCB. This section studies the mechanism of regulation of genes via steroid hormones and steroid receptors using techniques of chemistry and molecular biology. This transfer has thus provided more diversity to the overall cohesive theme of research in LMCB. The molecular biology studies on steroid hormone responsive transcription promoters complements and interacts well with similar studies on virus transcription promoters by Carter's group and mouse mammary gland hormone-responsive transcription promoters by Oka's group.

Research performed in LMCB has been recognized by various honors, awards and grants to individual members. B. Carter was awarded a substantial research grant from the Cystic Fibrosis Foundation for gene therapy of cystic fibrosis. T. Flotte was awarded the first Johns Hopkins-NIH Joint Cystic Fibrosis Foundation Fellowship in Pediatric Pulmonary Research. B. Antoni, on the basis of her work on HIV as an IRTA Fellow, was awarded an NRC Research Associateship. B. Carter was an Invited Plenary Speaker at the Fourth International Meeting on Parvoviruses in Israel and will be an Invited Symposium Speaker at the 1990 North American Cystic Fibrosis Annual Meeting. B. Antoni was an invited speaker at the International Parvovirus Meeting in Israel and was granted an EMBO Travel Award. B. Carter was appointed Chairman of the NIH Biosafety Committee, was reappointed for a fourth term to the Journal of Virology Editorial Board and continues to serve on the Parvovirus Subgroup of WHO-sponsored International Committee for Nomenclature of Viruses. I. Hinnant, a MARC Summer Student in 1989 received a MARC award for his research. Dr. T. Oka was an invited speaker at the Annual Meetings of the Japanese Biochemical Society, at the Japanese Polyamine Society, The FASEB American Physiological Society and the American Dairy Science Association.

Several members of the laboratory including T. Oka, N. Chejanovsky and B. Carter were invited to write a number of critical reviews in their fields.

Members of the Laboratory have maintained international collaborations with groups in Tokyo (Japan), Beersheva (Israel), Bet-Dagan (Israel), Tel Aviv (Israel) as well as collaborations with U.S. laboratories at Rutgers (N.J.), Toledo (Ohio) and Ann Arbor (Michigan).



## Function of DNA Virus Genomes in Animal Cells

The group led by B. Carter has continued to employ DNA viruses as molecular probes to study genome expression in human cells. The structure and function of adeno-associated virus (AAV) is being studied intensively. AAV has also been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes in mammalian cell chromosomes to yield stable expression. This vector also may be useful for therapy. The control of gene regulation in AAV vectors is being analysed in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. In a newly developed project, sponsored in part by the Cystic Fibrosis Foundation, we are attempting to develop vectors for possible gene therapy of cystic fibrosis.

A complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediate transcriptional activation and also translational inhibition of some genes. Site-directed mutagenesis is being used to resolve the functions of the rep gene. Coding of all these functions in a single gene appears to be unique in eukaryotic systems. Adenovirus is being studied since this is the helper virus for AAV multiplication. This helper relationship is being analyzed. Also, both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus, this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. The mechanism of this inhibition if tumor induction by AAV is being studied at the molecular level in both cell culture and in animal experiments.

The regulation of Human Immunodeficiency Virus (HIV) by AAV is being studied. Current work suggests that production of vaccines or use of nucleotide analog therapies for AIDS may be limited and difficult. Thus other therapeutic approaches are urgently required. Attempts are underway to develop a novel approach by using the negative regulatory property of a trans-acting gene of the human parvovirus, AAV, to inhibit the function of the trans-acting gene tat, of HIV. A functional tat gene is required for HIV growth so inhibition of tat presents a potentially useful approach to an antiviral therapy. Preliminary evidence shows that the AAV rep gene blocks growth of infectious HIV.

## Hormonal Regulation of Cell Growth and Differentiation

Epidermal growth factor (EGF) is produced in large amounts by the mouse submandibular gland. It is also present in such biological fluids as plasma, milk, urine and saliva. EGF is a potent mitogen for a wide variety of cells in culture but its function in the body needs to be elucidated. Dr. Oka's studies have demonstrated that EGF plays a key role in the development of the mammary gland during pregnancy and mammary tumorigenesis in female mice; in males it serves a role in spermatogenesis by stimulating the meiosis of spermatocytes. Studies have been continued to elucidate the physiological role of EGF by employing a variety of experimental approaches. These procedures, have provided valuable information concerning the function of EGF in the body. The concentration of EGF in the submandibular gland and plasma of female mice increased significantly during pregnancy and attenuation of this rise in EGF by sialoadenectomy and anti-EGF treatment resulted in increased rate of spontaneous abortion, suggesting that EGF is necessary for the normal course of pregnancy. EGF also has a physiological role in

maintaining the normal structure of the epidermis. EGF production is greatly reduced in diabetic mice.

More recent studies are analysing the synergistic actions of the hormones insulin, glucocorticoid and prolactin to regulate casein expression in the mouse mammary gland. Genomic clones of the mouse casein gene were isolated and the entire 6.8 kb gene as well as 6 kb of 5' flank and 10 kb of 3' flank were sequenced. Chimeric genes containing a reporter CAT gene fused to the casein promoter region can be transfected into mammary epithelial cells and show appropriate hormone responses. Additional studies have identified both positive and negative control elements.

#### Lysosomal Transport and Storage Disease

This work is being conducted by Dr. Frank Tietze. Degradation of cellular biopolymers such as proteins and polysaccharides takes place chiefly within the lysosome. The end-products of this degradation, viz., amino acids and monosaccharides, are presumed to exit the lysosome to the cytoplasm, where further metabolism or expulsion to the external medium occurs. To study the process of lysosomal transport, methods were developed to load lysosomes of various cells with amino acids (e.g., cystine, tyrosine) or with a specific monosaccharide (viz., sialic acid) and to measure their rates of egress from the organelle. Studies of cystine egress from lysosomes of human polymorphonuclear leukocytes and of tyrosine from cultured rat thyroid cell lysosomes have revealed these processes to be carrier-mediated and stereospecific. The further demonstration that no egress of cystine could be detected from similarly loaded lysosomes from patients with the inherited disorder cytinosis indicated that this storage disease is due to a congenital defect of a specific lysosomal carrier. Similar studies on the egress of sialic acid from fibroblast lysosomes have suggested strongly that impaired lysosomal transport underlies another lysosomal storage disorder, free sialic acid storage disease.

More recently, Dr. Tietze has demonstrated the biochemical defect in another disorder of sialic acid metabolism, sialuria, which appeared to be due to excess synthesis of sialic acid rather than defective transport. The biochemical defect has now been defined as a failure of the enzyme UDP-N-acetylglucosamine 2-epimerase to be feedback inhibited by CMP-sialic acid, the final end product of sialic acid synthesis. This leads to excess synthesis of free sialic acid. This suggests that sialuria may be a rare, and possibly unique example of a human mutation affecting the allosteric center of an enzyme.

#### Summary

Research in LMCB overall has followed a common theme of performing basic science on gene expression and also applying this to study of disease states. Thus recent work from this laboratory has identified the basic biochemical defect in one genetic disease (sialuria) illuminated a possible role of EGF deficiency in another disease (diabetes), and is aimed at developing therapeutic approaches to an infectious disease (AIDS) and a genetic disease (cystic fibrosis).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK, 57501-14

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Function of DNA Virus Genomes in Animal Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Barrie J. Carter	Chief, LMCB	LMCB:NIDDK
Other: Nor Chejanovsky	Visiting Associate	LMCB:NIDDK
Irving Miller	Biologist	LMCB:NIDDK
Beth Antoni	NRC Research Associate	LMCB:NIDDK
Roland Owens	IRTA Fellow	LMCB:NIDDK
John Smuda	IRTA Fellow	LMCB:NIDDK
Terry Flotte	NIH-Johns Hopkins CF Fellow	LMCB:NIDDK
Sandra Afione	Guest Worker	LMCB:NIDDK

## COOPERATING UNITS (if any)

J.P. Trempe (Toledo, OH), N. Chejanovsky (Israel), J. Tal (Beersheba, Israel),  
D.F. Klessig (Rutgers), E. Mendelson (Tel Aviv, Israel)

## LAB/BRANCH

Laboratory of Molecular and Cellular Biology

## SECTION

## INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.25

## PROFESSIONAL:

4.75

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of a human parvovirus, adeno-associated virus (AAV). AAV has been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper, the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes into mammalian cell chromosomes to yield stable expression. This vector also may be useful for gene therapy. We are now analyzing intensively the control of gene regulation in AAV vectors in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. We have discovered a complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediate transcriptional activation and translational inhibition of some genes. Site-specific mutagenesis is being used to resolve these functions. Coding of all these functions in a single gene is unique in eukaryotic systems. Adenovirus is the helper for AAV. This relationship is being analyzed. Both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus, AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in cell culture. We also are analyzing interactions of AAV with HIV as a potential approach to a novel therapy for AIDS. We are also developing AAV vectors that express the CFTR gene as a potential gene therapy for cystic fibrosis.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57502-17

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal Regulation of Cell Growth and Differentiation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Takami Oka	Senior Investigator	LMCB:NIDDK
Other:	Sergio Lavandero	Courtesy Associate	LMCB:NIDDK
	Chang-Soo Lee	Visiting Fellow	LMCB:NIDDK
	Shinzaburo Noguchi	Visiting Fellow	LMCB:NIDDK
	Yoshito Ohba	Visiting Fellow	LMCB:NIDDK
	John W. Perry	Biologist (Technician)	LMCB:NIDDK
	Katsuya Wada	Visiting Fellow	LMCB:NIDDK
	Masami Yoshimura	Visiting Associate	LMCB:NIDDK

## COOPERATING UNITS (If any)

Dr. Kishio Furuya, National Institute of Physiological Sciences, Japan  
 Dr. Koichi Enomoto, Shimane Medical University, Japan  
 Dr. Margot Ip, Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York

## LAB/BRANCH

Laboratory of Molecular and Cellular Biology

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.5

## PROFESSIONAL:

5.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The expression of casein gene in the mammary gland is stimulated by the synergistic actions of insulin, glucocorticoid and prolactin *in vitro* through enhancement of both transcription of the gene and stability of the transcripts. Our recent study suggests that protein synthesis is required for hormonal stimulation of casein gene transcription, but not for the increased stability of casein transcripts. To study the molecular mechanism of hormone action on casein gene expression, we have isolated the genomic clones of mouse beta-casein and the complete nucleotide sequence of the 6.8-kb casein gene and its immediate 6-kb (5') and 10-kb (3') flanking region has been determined. To study the regulatory sequence elements responsible for casein gene expression, we constructed a chimeric gene containing 5.3 kb of the 5' and 1.6 kb of the 3' flanking sequences of the mouse  $\beta$ -casein gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. The chimeric gene was transfected into primary mouse mammary epithelial cells prepared from pregnant mice. Expression of the  $\beta$ -casein-CAT chimeric gene required the synergetic actions of insulin, hydrocortisone and prolactin. Expression of the chimeric gene was also dependent on the appropriate substratum, since the degree of hormonal induction of the chimeric gene was much higher in cells cultured on a reconstituted basement membrane (Matrigel) than in cells cultured on either type I collagen gel or plastic. Additional transfection experiments using a series of  $\beta$ -casein-CAT constructs suggested the existence of positive and negative regulatory elements responsible for hormonal induction. We have extended our studies to investigate the possible role of EGF in other organs and also the control mechanisms involved in EGF gene expression and to identify diseases associated with EGF deficiency. Our studies indicate that in diabetic mice, the level of EGF and its mRNA in the submandibular gland as well as circulating EGF are greatly reduced and that insulin treatment corrects the defect in EGF production. The possibility that EGF deficiency is involved in manifestation of pathological complications in diabetes mellitus is currently investigated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57503-17

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Studies on the Metabolic Defect in Sialuria

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Frank Tietze Research Chemist LMCB:NIDDK

Other: None

COOPERATING UNITS (if any)

William A. Gahl, Human Genetics Branch, NICHD  
Gilbert Ashwell, Laboratory of Biochemistry and Metabolism, NIDDK

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Three human diseases of sialic acid metabolism (sialidosis, Salla disease and infantile sialic acid storage disease) result from defects in catabolism of sialic acid and lead to lysosomal storage. A fourth disorder of sialic acid metabolism, sialuria, has been postulated to result from defective regulation of sialic acid synthesis, in which the enzyme UDP-N-acetylglucosamine 2-epimerase (UDP-GlcNAc epimerase) fails to be feedback inhibited by CMP-sialic acid, the final end-product of sialic acid synthesis, leading to excess synthesis of free sialic acid. This unique inborn error, described thus far in only three patients, is characterized by variable degrees of developmental delay, coarse facial features and massive urinary excretion of unconjugated sialic acid. Cultured skin fibroblasts from patients with this disease contain greatly increased quantities of free sialic acid localized within the cytosolic compartment (50-100 nmoles/mg cell protein) in comparison with the amount found in normal fibroblasts (ca. 1 nmole/mg protein). We have explored the postulated role of defective feedback inhibition of UDP-GlcNAc epimerase activity in the pathogenesis of human sialuria by direct measurement of enzyme activity in cultured fibroblasts from all three known patients with sialuria and in normal subjects. Our studies have shown that 50  $\mu$ M CMP-sialic acid inhibited the UDP-GlcNAc epimerase of normal cells by 84-100% but inhibited the epimerase from sialuria cells by only 19-31%. We conclude that the basic biochemical defect in all known cases of sialuria is a failure of CMP-sialic acid to feedback inhibit UDP-GlcNAc epimerase. Sialuria may represent a rare, and possibly unique, example of a human mutation affecting an enzyme allosteric center.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57504-03

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Regulation of HIV by AAV

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Barrie J. Carter Chief, LMCB LMCB:NIDDK  
Other: Irving L. Miller Biologist LMCB:NIDDK  
Beth Antoni IRTA Fellow LMCB:NIDDK

COOPERATING UNITS (if any)

A.S. Rabson (LMM, NIAID)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.25

PROFESSIONAL:

1.25

OTHER:

-

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The etiologic agent of AIDS is the human immunodeficiency virus (HIV) which differs from most other human viral diseases in exhibiting a very prolonged latent period, but ultimately being lethal due to a profound effect on the immune system. Several trans-acting HIV genes appear to be crucial to HIV growth and infection. Therefore we are studying the feasibility of a novel anti-viral therapy for HIV based on interference by another viral gene with the trans-acting regulation of HIV. The overall goal of this proposal is to analyze interactions between trans-acting regulatory genes of HIV and of a human parvovirus, adeno-associated virus, AAV. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. Current work suggests that developing standard types of anti-viral therapy such as vaccines or nucleotide-analog drugs for HIV is difficult and other alternate possibilities for therapy must be investigated. One approach is to intervene in the trans-regulation system of HIV especially that mediated by the HIV tat gene. Thus a possible anti-viral therapy for HIV is to inhibit the production or the action of tat. A novel way to attempt this is to employ a trans-acting gene from another human virus. One such candidate is the rep gene of the human parvovirus adeno-associated virus (AAV). AAV does not cause any human disease and grows only in cells also infected with adenovirus or herpes viruses. AAV inhibits growth of the helper virus and may play an important role in limiting certain human viral infections. Also AAV can alter important regulatory controls in virus infected cells or in tumor cells. Rep is a novel type of trans-acting regulatory gene which exhibits negative, translational regulation of many genes in several cell types. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. We are testing rep as a potential

ANNUAL REPORT OF THE  
LABORATORY OF ANALYTICAL CHEMISTRY,  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Analytical Chemistry has consisted of an Office of the Chief and five Sections, each operating rather independently in terms of research objectives and activities. Following the retirement of Dr. David Johnson as Laboratory Chief, a decision was made by NIDDK to reorganize the Laboratory to concentrate on its mission of providing specialized analytical services and instrumentation, together with other support services for NIDDK. Research in new analytical methodologies, together with collaborative research on a variety of topics related to the Laboratory's role, will continue. In addition, LAC personnel continue to be responsible for overseeing safety and other services for Building 8 and for maintaining the building library and chemical stores. However, the research activities of three of our Sections are in process of being transferred, in accordance with the wishes of the Sections, to other Laboratories of NIDDK. The Medicinal Chemistry Section will move to the Laboratory of Structural Biology and will be renamed the Natural Products Section; the Biomedical Chemistry Section will become part of the Laboratory of Medicinal Chemistry; and the Steroid Hormones Section will move to the Laboratory of Molecular and Cellular Biology. This is the final report of the Laboratory of Analytical Chemistry as it was constituted prior to the reorganization.

#### INSTRUMENTATION SECTION

This Section provides analytical services, including elemental analyses (carried out under contract) and the use of sophisticated instrumental methods, primarily nuclear magnetic resonance (NMR) and mass spectroscopy (MS). New and refined methods are often developed to improve the facility to provide services and as part of various collaborations, as described below. (H. J. C. Yeh, N. F. Whittaker, W. L. White, E. D. Becker; also L. K. Pannell, LBC.)

##### Applications of NMR in Biochemical and Biological Systems (H. J. C. Yeh):

NMR structural assignment and conformational study of molecules with submilligram quantity and/or with congested resonance peaks often require sophisticated experiments (such as 2D Relayed COSY, HMQC etc.) carried out at the highest possible magnetic field strength for gaining sensitivity and resolution. Using these methods on our recently acquired high field NMR spectrometer (Varian 500), a number of compounds provided by other investigators has been investigated and their structures have been determined. This includes 1) submilligram quantity of nucleotide adducts of diol epoxides derived from polycyclic aromatic hydrocarbons (D. Jerina and J. Sayer), 2) alkaloids, such as danazol (E. Gros and M. Garraffo), multiflorines (M. Wysocka and T. Brukwicki), colchinoids (A. Brossi) and toxins from extracts of frog skin (J. Daly and T. Spande), 3) polysaccharides (P. Kovac) and 4) ketodienes from culticular lipids of the Guam Brown Tree Snake (R. Mason, Y. Murata, L. Pannell, and T. Jones).

The CPMG  $T_2$  experiment is a useful technique in obtaining dynamic and structural information of molecules. Recently, it has been reported that the short  $^{19}\text{F}$  NMR spin-spin relaxation time ( $T_2$ ) for halothane in brain could be of pharmacological relevance. Since these findings appeared to contribute significantly to an understanding of the molecular actions of anesthesia, an area that remains controversial, we have carried out similar studies in brains and in other tissues as well. We find that the highly immobilized environment for halothane as characterized by the short  $T_2$  spin-spin relaxation time is not unique to brain. Short  $T_2$  environments for halothane were also obtained in packed red blood cells and other peripheral tissues (e.g. liver and kidney) of anesthetized rats. While the presence of the short  $T_2$  for halothane in brain (and other tissues) may be of interest, our present findings indicate it is not directly related to the molecular mechanisms of anesthesia (E. Moody and P. Skolnick).

#### Utilization of MS in Chemical Analyses (N. F. Whittaker):

Iodoimidazole type compounds synthesized by Dr. Avramovici and Dr. Cohen have been successfully analyzed in the electron impact mode after introduction via a liquid chromatograph. Work is underway with Dr. Hubel and Dr. Kirk of the LBC to access the potential for detecting amino acid mixtures by particle beam in the chemical ionization mode. A sensitive method for detection of amino acids separated by liquid chromatography would be of great value. This technique has also proved useful in a variety of other applications.

Electron impact accurate mass capabilities of the VG mass spectrometer have been used extensively in a cannabinoid research project with Dr. Richardson and Dr. Rice. Because the synthesized compounds are frequently oils, making them difficult to characterize by traditional CHN analysis, accurate mass determination of the molecular ions is necessary. Accurate mass measurement is an important analytical technique for oils or samples available only in microgram amounts. Work is in progress with Dr. Kovac of the Carbohydrate section of the LMC to obtain chemical ionization spectra of oligosaccharides in the mass range of one to two thousand.

#### STEROID HORMONES SECTION

Work in this section is aimed at defining the initial, intracellular events of glucocorticoid hormone action, and steroid hormone action in general. The first step of steroid binding to the intracellular receptor molecule is followed by activation of the receptor-steroid complex to a DNA-binding and nuclear-binding species and then binding of activated complexes to those nuclear acceptor sites involved in the regulation of transcription of specific genes. A combination of techniques have been used to examine the crucial first step for glucocorticoid steroids. Studies with the affinity label dexamethasone mesylate (Dex-Mes) and various proteases have defined a steroid-binding core fragment that is half the size of the previously acknowledged minimum steroid binding fragment. The sequence of this core fragment was found to be Thr<sup>537</sup> - Arg<sup>673</sup>. Studies with arsenite (and  $\text{Cd}^{++}$ ), which specifically reacts with vicinal dithiols, and the affinity label Dex-Mes have confirmed the involvement of our previously proposed vicinal dithiol group in



steroid binding to the glucocorticoid receptor. Further studies of proteolytic fragments of the receptor using these thiol-specific reagents, Dex-Mes, and a specific anti-receptor antibody, revealed that, of the 20 cysteines in the receptor, only the 3 closely spaced cysteines in the steroid binding core are candidates for being the vicinal dithiols. Further studies using these techniques, along with molecular biology, should provide new information about steroid interactions with the receptor protein at a molecular level. This information will be invaluable in understanding the determinants for glucocorticoid vs antiglucocorticoid hormone action at the level of gene transcription. (S. S. Simons, Jr., A. Cavanaugh, H. Oshima, P. Chadrabarti, Y. Miyashita, D. Szapary.)

## BIOMEDICAL CHEMISTRY SECTION

### Advances on Understanding the Interferon-Induced 2-5A System:

2',5'-oligoadenylates known as 2-5A [ $p_x(A_2'p)_nA$ ;  $x = 2$  or  $3$ ,  $n \geq 2$ ] are produced in interferon-treated cells in response to double-stranded RNA. 2-5A binds with high affinity to a 2-5A-dependent RNase resulting in the cleavage of single-stranded RNA. An efficient, rapid, and extremely sensitive photoaffinity labeling method was developed to facilitate detection of 2-5A-dependent RNase. A bromine-substituted and radioactive derivative of 2-5A, the 5'-monophosphate,  $p(A_2'p)_2[(b^8A)2'p]_2A_3'-[^{32}P]C_p$ , was synthesized as a probe for 2-5A-dependent RNase. Even though this bromine-substituted analog of 2-5A bore no 5'-terminal triphosphate or diphosphate, it bound to 2-5A-dependent RNase with the same high affinity as did 2-5A per se, but it was a less effective activator of the RNase under the assay conditions. The presence of bromine atoms in the 2-5A analog enhanced by more than 200-fold crosslinking to 2-5A-dependent RNase under a uv lamp; many additional polypeptides were also labeled but at much lower levels. Furthermore, using high-intensity uv laser irradiation (308 nm), covalent attachment of the bromine-substituted 2-5A analog to 2-5A-dependent RNase was readily achieved within  $10^{-6}$ s.

NMR and model-building studies were carried out on  $pA_2'5'A_2'5'A$  and analogs in which one or more of the A residues were replaced by 8-bromoadenosine. Chemical shifts, coupling constants and NOE data were used to obtain structural information. The N/S equilibrium constant of the ribose rings as well as the phase angles and puckering amplitudes were determined from the experimental coupling constants with the aid of an improved version of the PSEUROT program. Chemical shifts in combination with NOE data were used to monitor base-base interactions and the orientation of the bases (*syn* or *anti*)

The combined data suggest that different types of stacking interactions are present in the various compounds. Bromination of the first or second residue in the trimers results in a preference for N-type sugar and *syn* orientation of the base in these residues. When A(3) is brominated, an S-type sugar conformation together with a *syn* orientation of the base is favored at the 2' terminus. Energy-minimized models of the different stacking interactions were developed and a correlation between the biological activity of these compounds and their conformation was suggested.

A number of novel 2',5'-oligoadenylate analogs have been synthesized using a solid phase methodology developed in this laboratory. These analogs include a variety of sequence-specific

fluororibose analogs as well as base-modified 2'5' oligonucleotides bearing sequence-specific substitutions of uracil, cytosine and guanine bases. In addition an analog has been prepared with only a hydrocarbon chain spacer in place of the second adenosine moiety of 2-5A trimer. These analogs are being employed to probe the active site of the 2-5A-dependent endonuclease, RNase L. (P. F. Torrence, K. Lesiak, T. Kovacs.)

#### 3'-Amino-3'-Deoxythymidine 5'-Triphosphate as an Inhibition of Human Immunodeficiency Virus Reverse Transcriptase:

In collaborative studies with Keder, Abbotts and Wilson of NCI, we established that 3'-amino-3'-deoxythymidine 5'-triphosphate is a potent non-competitive inhibitor of HIV reverse transcriptase in contrast to 3'-azido-3'-deoxythymidine 5'-triphosphate which is a potent competitive inhibitor of the enzyme. Since the non-competitive nature of this inhibition with the amino analog may suggest a new lead to nucleotide inhibitors of HIV reverse transcriptase, we have initiated studies on the conformation of 3'-amino-3'-deoxythymidine itself to determine if this molecule possesses any unusual features that could contribute to this inhibiting behavior. (P. F. Torrence, K. Lesiak, T. Kovacs, S. Khamnei.)

### MEDICINAL CHEMISTRY SECTION

#### Physostigmine and Analogs:

Novel carbamates of the (-)-N(1)-series proved highly potent. Synthesis of ( $\pm$ )-physoverine has been achieved and is being extended to a synthesis of the natural alkaloid. A synthesis of natural N(8)-norphysostigmine from 5-methoxy-N-methyltryptamine has been successfully reinvestigated (M. Browstowska, Y. Sekine, A. Brossi).

#### Mammalian Alkaloids:

Methylation of (S)- and (R)-isoquinolines by (S)-adenosyl-L-methionine in the presence of mammalian catechol transferase showed only the (S)-enantiomers to be O-methylated to alkaloids useful for the biosynthesis of (S)-reticuline and morphine. The unnatural (R)-isomers were methylated differently. It is believed possible that man converts properly configured isoquinolines into mammalian morphine in a similar fashion to the biosynthesis used by the poppy plant and by using similar enzymes. (M. Browstowska, D. Tadic, A. Brossi).

#### Tubulin Binding:

It is now clearly established that all 4 methoxy groups in colchicine are important for binding. The N-acetamido group is not necessary, and the methoxy group at C(10) of the tropolonic ring can be replaced by a thiomethyl group or an amino group. Optically active allo-compounds with the N-acetamido group at C(5) did not inhibit tubulin polymerization in vitro. So far no useful marker of the colchicine binding site on tubulin has been discovered (O. Boyé, A. Brossi).

#### Nortropane Alkaloids:

The Chinese alkaloid baogongteng A from Erycibe plants has medically useful cholinergic properties. A total synthesis of the natural alkaloid is being attempted from readily available 6-hydroxytryptan-2-one. Chemical resolution of the racemic material has been achieved. Introduction of the 2-hydroxy group will be first tried by bromination of a properly protected nor-analog which has been prepared, and replacing the bromine with a hydroxy group using silver carbonate (X.-S. He, A. Brossi).

#### Analytical Reagents from Dihydrofluorescein:

O-Benzylidihydrofluorescein, obtained from fluorescein by benzylation, reduction and alkaline hydrolysis, proved to be a versatile intermediate to prepare alkyl- and acyl-substituted analogs. O,O-Dibenzylidihydrofluorescein (DBDF) emerged as a useful reagent for derivatizing amines and alcohols.

Conversion into red dyes is accomplished after isolation by catalytic reduction and oxidative iodination. The lead is further explored to design optically active representatives (D. Tadic, A. Brossi).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58000-45 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analytical Services and Methodology

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. D. Becker	Acting Chief, Lab. Anal. Chem.	LAC/NIDDK
OTHER:	H. J. C. Yeh	Research Chemist	LAC/NIDDK
	N. Whittaker	Chemist	LAC/NIDDK
	W. White	Biologist	LAC/NIDDK
	L. K. Pannell	Research Chemist	LAC/NIDDK

## COOPERATING UNITS (if any)

Laboratories of Chemistry, Bioorganic Chemistry and Medicinal Chemistry, NIDDK

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Instrumentation Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists of the Laboratory of Chemistry, Laboratory of Bioorganic Chemistry, other NIH laboratories and, to a limited extent, to personnel of other government agencies. Instrumental analyses include GC/MS spectrometry, HPLC/MS spectrometry, infrared spectrometry, nuclear magnetic resonance spectrometry, and element detection using a microwave plasma detection system. Assistance in interpretation of spectra is rendered on request. Samples for elemental microanalysis are handled through external contracts.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58001-17 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Application of NMR in Biochemical and Biological Systems

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H. Yeh

Research Chemist

LAC/NIDDK

OTHER:

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Instrumentation

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Z01 DK 58001-17 has been combined with Z01 DK 58000-45 LAC.

Project terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58002-15 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Initial Intracellular Events of Steroid Hormone Action

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. S. Simons, Jr.	Chief, Steroid Hormones Section	LAC/NIDDK
OTHER:	A. Cavanaugh	Staff Fellow	LAC/NIDDK
	H. Oshima	Visiting Fellow	LAC/NIDDK
	P. Chakraborti	Visiting Associate	LAC/NIDDK
	Y. Miyashita	Visiting Fellow	LAC/NIDDK
	D. Szapary	Visiting Associate	LAC/NIDDK

## COOPERATING UNITS (if any)

E. Brad Thompson (Univ. of Texas, Galveston); B. Groner (Basel, Switzerland); Jeffrey M. Harmon (USUMS, Bethesda); J. D. Ashwell (NCI/NIH).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Steroid Hormones

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.4

## PROFESSIONAL:

6.0

## OTHER:

1.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to define the initial, intracellular events of glucocorticoid hormone action and steroid hormone action in general. The first step of steroid binding to the intracellular receptor molecule is followed by activation of the receptor-steroid complex to a DNA-binding and nuclear-binding species and then binding of activated complexes to those nuclear acceptor sites involved in the regulation of transcription of specific genes. A combination of techniques have been used to examine the crucial first step for glucocorticoid steroids. Studies with the affinity label dexamethasone mesylate (Dex-Mes) and various proteases have defined a steroid-binding core fragment that is half the size of the previously acknowledged minimum steroid binding fragment. The sequence of this core fragment was found to be Thr-537 - Arg-673. Studies with arsenite (and Cd<sup>++</sup>), which specifically reacts with vicinal dithiols, and the affinity label Dex-Mes have confirmed the involvement of our previously proposed vicinal dithiol group in steroid binding to the glucocorticoid receptor. Further studies of proteolytic fragments of the receptor using these thiol-specific reagents, Dex-Mes, and a specific anti-receptor antibody, revealed that, of the 20 cysteines in the receptor, only the 3 closely spaced cysteines in the steroid binding core are candidates for being the vicinal dithiols. Further studies using these techniques, along with molecular biology, should provide new information about steroid interactions with the receptor protein at a molecular level. This information will be invaluable in understanding the determinants for glucocorticoid vs antiglucocorticoid hormone action at the level of gene transcription.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK.58003-17 LAC

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Methods and Materials for the Study of Medical Problems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. M. Foltz

Research Chemist

LAC/NIDDK

OTHER: B. Baer

Chemist

LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Steroid Hormones

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The primary goal of this work is to contribute to the investigation and solution of basic medical problems by the application of chemical, physical and biological methods. This goal is being pursued by studies of the biology and biochemistry of murine tumor cells with emphasis on cancer metastasis.

Studies were begun several years ago to determine whether one or more specific gene products are required to confer on certain tumor cells the properties needed for cancer metastasis. Promising preliminary results obtained with NIH 3T3 cells transfected with one of several oncogenes were reported previously. This work has been suspended temporarily because of other priorities.

Other lines of work in the area of cancer metastasis, such as the interactions of tumor cells with basement membrane components and other biological materials, the nutrition of tumor cells, and the effects of the treatment of tumor cells with chemicals and biologicals on metastatic potencies, are also to be resumed when the priorities imposed by other responsibilities permit. Meanwhile, and several lines of murine tumor cells, which have been used in the past and which are to be used in the future, are being maintained in mice and their metastatic potencies monitored.

During this period C. M. Foltz has been serving as Safety Officer for Building 8/8A, and Manager of Building 8/8A, and much of his activity has been in those areas.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58004-23 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Professional Practices of Biomedical Scientists

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: N. Feder

Medical Officer (Research)

LAC/NIDDK

OTHER: W. W. Stewart

Research Physicist

LAC/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Biophysical Histology

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies have continued on the professional practices of biomedical scientists and on the accuracy of the scientific literature.

The studies on neuronal structure in isogenic snails and on the synthesis of a new rhodamine dye have been discontinued.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58005-17 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. F. Torrence.	Research Chemist	LAC/NIDDK
OTHER:	K. Lesiak	Visiting Scientist	LAC/NIDDK
	T. Kovacs	Visiting Fellow	LAC/NIDDK

## COOPERATING UNITS (if any)

R. Silverman, Dept. Pathology, USUHS, Bethesda, MD; P. Herdefiwn, Rega Institute, Leuven, Belgium.

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Biomedical Chemistry

## INSTITUTE AND LOCATION

NIDDK NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL

3.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |   |                                      |
|---|---|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input checked="" type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |   |                                      |
| <input type="checkbox"/> (a2) Interviews    |   |                                      |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interferon-induced enzyme activities such as the oligo (2'5') adenylate synthetase, and the oligo (2'5') A phosphodiesterase are investigated with the goal of understanding their role in the action of interferon, the induction of interferon by double-stranded RNA and, perhaps, control of cell growth and differentiation. Analogs of the mediator of interferon action, 2-5A, are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A dependent endonuclease. The eventual goal is to understand the biological role of the 2-5A system and to explore the potential of exploitation of this system in chemotherapy.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58006-07 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry and Metabolism of Qinghaosu: A Chinese Antimalarial Drug

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Brossi	Visiting Scientist	LAC/NIDDK
OTHER:	Q. S. Yu	Visiting Fellow	LAC/NIDDK
	A. Muzaffar	Visiting Fellow	LAC/NIDDK

## COOPERATING UNITS (if any)

Walter Reed Army Institute of Research, Washington, D. C., NIH (W. Milhous); Laboratory for the Structure of Matter, Department of the Navy, Washington, D. C., (J. Flippen-Anderson).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Structure of a dimer obtained from deoxydihydroartemisinin by acid catalyzed dehydration was established by X-ray analysis.

Project terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58007-06 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Physostigmine and Analogs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Brossi	Visiting Scientist	LAC/NIDDK
OTHER:	Y. Sekine	Volunteer	LAC/NIDDK
	V. Pabuccuoglu	Visiting Fellow	LAC/NIDDK
	M. Brzostowska	Visiting Fellow	LAC/NIDDK

## COOPERATING UNITS (if any)

NIAID, NIH (Dr. Timothy Soncrant and S. I. Rapoport); Institute of Organic Chemistry, Academia Sinica, Shanghai, China (Dr. Quiang-shen Yu).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.3

PROFESSIONAL:

1.3

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Novel carbamates of N(1)-norphysostigmine of the (-)-series were prepared. Both, the butyl- and the octyl-analog were found to be highly potent in vitro in inhibiting cholinesterases. Synthesis of (+)-physovenine from intermediates of the physostigmine synthesis was accomplished. Synthesis of optically active material by phase-transfer induced chirality has been achieved. Synthesis of natural N(8)-norphysostigmine from 5-methoxy-N-methyltryptamine has been initiated and led to (+)-N(8)-noreseroline methyl ether. Resolution by the phenylethylurea method is planned.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58010-05 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Mammalian Alkaloids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator; Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

OTHER: Y. Sekine

Volunteer

LAC/NIDDK

D. Tadic

Visiting Fellow

LAC/NIDDK

## COOPERATING UNITS if any:

Laboratory of Bioorganic Chemistry, NIDDK (C. Creveling); Dept. of Psychiatry, School of Medicine, Vanderbilt University, Nashville, TN (S. Spector).

## LABORATORY

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSORIAL

OTHER

## CHECK APPROPRIATE BOXES

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard nomenclature type. Do not exceed the space provided.)

Only S-norcodeine and S-4'-demethylnorcodeine are converted with SAM in the presence of COMT into O-methylated congeners of the reticuline biosynthetic pathway. The corresponding R-enantiomers are methylated at different hydroxy groups. This adds more evidence to the theory that mammalian morphine may derive from the same precursor isoquinolines, (S)-norcodeine and (S)-reticuline, utilized by the poppy plant and by using the same enzymes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK58011-14 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Structure-Activity Relationships of Colchicins Based on Tubulin Binding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, address, and institute affiliation.)

PI:	A. Brossi	Visiting Scientist	LAC/NIDDK
OTHER:	O. Boyé	Visiting Fellow	LAC/NIDDK
	A. Muzaffar	Visiting Fellow	LAC/NIDDK

## COOPERATING UNITS (if any)

Division of Cancer Treatment, National Cancer Institute, NIH (E. Hame); Hebrew University Jerusalem, Dept. of Pharmacology (I. Ringel); Palacky University, Olomouc, Czechoslovakia, Med. Chemistry (V. Simanek).

## LABORATORY

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland, 20892

## TOTAL MAN-YEARS

2.2

## PROFESSORIAL

1.1

## OTHER

## CHECK APPROPRIATE BOXES:

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Amides of deacetylcolchicine and deacetylisocolchicine with dihydrofluorescein diacetate have been prepared as potential markers of the colchicine binding site on tubulin.  $^{14}\text{C}$ -labeled isothiocyanato-deacetylthiocolchicine did not prove to be useful to specifically mark the colchicine binding site. Total synthesis of two ring A dimethoxy substituted dibenzocycloheptatrienes revealed that all 3 methoxy groups in the ring A of colchicine are important for the interaction with proteins. Optically active isomers of N-acetylcolchinal methyl ether with the acetamido group at C(5) did not bind to tubulin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK-58012-04 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Antiviral Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

OTHER: V. Pabuccuoglu

Visiting Fellow

LAC/NIDDK

## COOPERATING UNITS (if any)

School of Pharmacy, University of Mississippi, University, MD (A. Clark and C. D. Hufford).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The phenolic oxoaporphine alkaloids liriodendronine and its 2-O-methyl ether were prepared from lysicamine by O-demethylation with pyridinium hydrobromide at 200°C and in refluxing 48% hydrobromic acid respectively. Quaternary lysicamine methiodide undergoes decomposition in refluxing acetone. Salts of phenolic oxoaporphines are converted, on treatment with pyridine-water, into highly colored quinone-methides. The solvent system pyridine-alcohol-methylene chloride proved extremely useful to investigate chemical purity of phenolic oxoaporphines by tlc-analysis. Quinonemethides could be crystallized from pyridine-water. This project has been completed.

Project terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58013-04 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Beta-Carbolines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

OTHER: L. Chrisey

Visiting Fellow

LAC/NIDDK

## COOPERATING UNITS (if any)

School of Pharmacy, Department of Medicinal Chemistry, University of Texas at Austin (C. W. Abell).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

PROFESSIONAL

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Optically pure tetrahydroharmine was prepared by chemical resolution and optical purity was assessed by HPLC-analysis of ureas obtained with an optically active isocyanate. Optically active tetrahydroharmine racemizes on standing in acidic solution. Several anhydronium bases of beta-carbolines were prepared and are fully characterized. This project has been completed.

Project terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58014-03 LAC

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. F. Torrence.	Research Chemist	LAC/NIDDK
OTHER:	K. Lesiak	Visiting Scientist	LAC/NIDDK
	T. Kovacs	Visiting Fellow	LAC/NIDDK
	S. Khamnei	Visiting Fellow	LAC/NIDDK

COOPERATING UNITS (if any)

FOREIGN: Rega Institute, Catholic University of Leuven, Belgium (Dr. E. DeClercq and J. Balzarini); Nagoya City University, Japan (Dr. K. Kohda). NIH: Dr. N. Greig, NIMH; Dr. S. Wilson, NCI. Dr. K. Pannell (Univ. Texas, El Paso), Dr. I. Pelczer (Univ. Syracuse).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Biomedical Chemistry

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland, 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The nucleoside, 3'-amino-3'-deoxythymidine, has been found to be a potent, linear, non-competitive inhibitor of human immunodeficiency virus reverse transcriptase. As a potential lead to understanding the mechanism of inhibitor of HIV reverse transcriptase by nucleotide analogs, the conformation of the amino-nucleoside has been investigated by NMR and X-ray. In the solid state, a rare syn conformation is assumed by the glycoside; in solution, the normal anti conformation is observed. The hydrogen bonding capacity of the 3'-amino moiety relates to the unusual crystal conformation and perhaps to the unusual HIV reverse transcriptase inhibitory behavior. Poly(2'-fluoroadenylic acid), a potent inhibitor of Maloney murine leukemia virus reverse transcriptase, fails to inhibit the HIV reverse transcriptase.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58015-03 LAC

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oxindoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

OTHER:

COOPERATING UNITS (if any)

Institute of Organic Chemistry, Academia Sinica, Shanghai, China (Dr. Q. S. Yu).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(+)-Physovenine present in Calabar beans has been obtained in the form of a racemic mixture by synthesis from an oxindole intermediate of the physostigmine synthesis.

Z01 DK 58015-02 LAC terminated and combined with Z01 DK 58007-06 LAC.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58016-02 LAG

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhibition of Vesicular Stomatitis Virus RNA Polymerase by 2'5'-Oligoadenylates

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. F. Torrence.	Research Chemist	LAC/NIDDK
OTHER:	K. Lesiak	Visiting Scientist	LAC/NIDDK
	T. Kovacs	Visiting Fellow	LAC/NIDDK

## COOPERATING UNITS (if any)

J. Lenard, Robert Wood Johnson Medical School, WMDNJ, Piscataway, NJ.

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Biomedical Chemistry

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The diadenylate triphosphates, ppp5'A2'p5'A and ppp5'A3'p5'A, were found to inhibit the purified RNA polymerase ("nucleocapid") complex from vesicular stomatitis virus (vsv). This work has been completed.

Project terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58017-01 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Nortropane Alkaloids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

OTHER: X-S. He

Visiting Fellow

LAC/NIDDK

## COOPERATING UNITS (if any)

National Institutes of Pharmacological Research and Development, Beijing, China (Dr. C. C. Shen).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Synthesis of Chinese nortropane alkaloid boagongteng A from Erycibe plants has been initiated from (+)-6-hydroxytropin-2-one. Optical resolution has been accomplished with camphersulfonic acids. N-demethylation and N-benylation afforded (+)-6-acetoxy-N-benzyltropin-2-one which will be used in the next step. N-Demethylation of tertiary amines with trichloroethyl chloroformate gives ditrichloroethyl carbonate as a by-product.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK58018-01 LAC

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analytical Reagents from Dihydrofluorescein

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

OTHER: D. Tadic

Visiting Fellow

LAC/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Ethers and acetates of dihydrofluorescein have been developed as analytical reagents. The dibenzylether of dihydrofluorescein seems particularly useful for derivatizing amines and alcohols. Reduction of dihydrofluoresceins in acetic anhydride over  $\text{Pd}(\text{OH})_2$  catalyst converts the carboxy group into a methyl group, reminiscent of the Rosenmund reduction of acyl chlorides to aldehydes.

## ANNUAL REPORT OF THE LABORATORY OF NEUROSCIENCE

### NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

#### Studies on the benzodiazepine/GABA receptor chloride channel complex

The benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") is an oligomeric group of proteins that contains recognition sites for many psychopharmacological agents including benzodiazepines, beta-carbolines, barbiturates, and "cage" convulsants (such as picrotoxin). The proteins comprising this complex act in concert to regulate the activity of chloride channels that are controlled ("gated") by gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the vertebrate central nervous system. Studies are in progress to characterize the molecular aspects of this system, its physiological functions and possible role in disease.

Molecular Aspects The supramolecular complex appears to be one member of a "superfamily" of ligand-gated ion (both cation and anion) channels which may include channels gated by glycine (anion), acetylcholine (cation), and glutamate (cation). While the supramolecular complex is currently thought to consist of three distinct but related proteins (termed alpha, beta and gamma), neither the number nor arrangement of subunits required to form a fully functional drug and ligand-gated chloride channel is known. Studies with photoactive 1,4-benzodiazepines have demonstrated an irreversible labelling of one predominant (alpha) species. However, molecular modelling studies indicate the photolabile moieties of these 1,4-benzodiazepines are located >6 Å from the proposed "active site" required for high affinity binding of these compounds. Moreover, molecular modelling studies indicate that high affinity binding of beta-carbolines would not necessarily require binding to completely identical residues. Rather, a common binding domain for all ligands together with an additional two or three areas for specific ligand-receptor interactions has been proposed. The synthesis of the 3- and 6-isothiocyanato derivatives of beta-carboline (referred to as 3- and 6-NCS-beta-carboline, respectively) together with the demonstration that they irreversibly inhibit ligand binding to benzodiazepine receptors suggests these compounds provide a means of testing this hypothesis if they can be prepared in a radioactive form with high specific activity. Both biochemical and electrophysiological evidence suggests that "cage" convulsants act at sites in or near GABA-gated chloride channels. The exact locus of action of such compounds is important in determining the molecular arrangement of the supramolecular complex as well as more precisely defining the site of action for depressants such as the barbiturates. Neurochemical studies of the ortho, meta and para-isothiocyano derivatives of t-butylbicycloorthobenzoate (B. daCosta and K. Rice) indicate that the latter two compounds act as acylators at GABA-gated chloride

channels while the former substance does not. However, molecular modelling of adducts formed by these compounds and a lysine residue demonstrated that the meta- and para- isomers are 30 and 47% longer than the ortho derivative respectively, in their minimum energy conformations. These findings, together with studies employing radioactive derivatives of these compounds should aid in defining the locus of action of cage convulsants. While diverse anesthetics, including inhalation agents perturb the supramolecular complex, studies using  $^{19}\text{F}$  NMR indicated unique, saturable binding sites in brain for the inhalation agent halothane. The demonstration that this apparent saturability resulted from halothane depressing respiratory rates together with a highly immobilized environment for this anesthetic that is not unique to brain suggests that  $^{19}\text{F}$  NMR techniques may not be applicable to quantitative molecular analysis of anesthetic action.

Pharmacological Aspects Previous studies from this laboratory have demonstrated that systematic modification of a beta-carboline (ZK 93423) with agonist actions resulted in derivatives with antagonist/inverse agonist properties. These findings led to the formulation of a pharmacophore for benzodiazepine receptor agonists containing three sites of electron density and two of lipophilicity. An excluded volume analysis of chemically diverse benzodiazepine receptor ligands resulted in the synthesis of a beta-carboline (SPH 509) with high affinity for benzodiazepine receptors which possesses a mixed agonist/antagonist profile. This compound is an efficacious anxiolytic/anticonvulsant but lacks muscle relaxant activity and can block the myorelaxant actions of diazepam. The ability to quantitate ligand binding to benzodiazepine receptors with fluorescent probes was previously reported. Further studies indicate benzodiazepine receptor ligands containing fluorescent moieties can also be employed for direct receptor visualization in thin tissue sections of the central nervous system. Moreover, conjugation of the benzodiazepine receptor ligand Ro 15-1788 with 7-nitrobenz-2-oxa-1,3-diazol-4-yl results in a ligand whose fluorescence quenches in a predicting manner upon binding to benzodiazepine receptors. This substance permits facile study of ligand-receptor interactions in real time.

Physiological role and implications in disease Previous studies indicate that many of the neurological manifestations in two different animal models of hepatic encephalopathy (HE) can be blocked by administration of benzodiazepine receptor antagonists such as Ro 15-1788 (flumazenil), and that extracts from the brains of these models contain higher concentrations of benzodiazepine receptor ligands. Elevated levels of N-desmethyldiazepam and diazepam have now been positively identified in both animal models using mass spectroscopy. Both the origin of these materials and identification of such substances in the human condition are experimental questions which must be addressed. We previously demonstrated that administration of anxiogenic beta-carbolines are

immunosuppressive, implicating the supramolecular complex in the neural control of immunity. Two lines of mice that were selectively bred for differential reactivities to drugs known to act at the supramolecular complex (e.g. ethanol, barbiturates, benzodiazepines) have now been shown to differ in several measures of immune function both before and after allogeneic priming. These observations provide further evidence for a central control of immune function (see below).

#### Studies on glycine and glutamate coupled cation channels

We previously demonstrated that  $Mg^{2+}$  and other substances known to interact with NMDA-gated cation channels can modulate strychnine-insensitive  $[^3H]$ glycine binding. We examined the ability of constrained glycine analogs to interact with these strychnine-insensitive glycine binding sites, and found that 1-aminocyclopropanecarboxylic acid (ACPC) is a specific, high affinity ligand for these sites. ACPC appears to be a partial agonist (compared to glycine) at these sites since it is not as efficacious in enhancing  $[^3H]$ MK-801 binding to NMDA-gated cation channels. Since electrophysiological studies indicate that glycine may be required for (rather than merely augment) NMDA-gated channel opening, the ability of ACPC to modulate NMDA-mediated events was examined both *in vivo* and *in vitro*. Like competitive NMDA antagonists, ACPC elicits an anticonflict action in the elevated plus-maze, an animal model of anxiety. The finding that ACPC mimicks the effects of clinically effective anxiolytics in a non-conflict model (ultrasonic vocalization in rat pups) of anxiety together with a reversal of this effect by glycine supports the notion that ligands at strychnine-insensitive glycine receptors may be clinically effective anxiolytics. Additional findings that ACPC, a cation channel blocker (MK-801), and a competitive NMDA antagonist (AP-7) all mimic clinically effective antidepressants in two animal models suggest that glutamatergic pathways may be involved in the pathophysiology of affective disorders. The ability of ACPC to attenuate NMDA-induced excitotoxicity in the embryonic chick retina indicates this and related compounds may be useful in ameliorating neurodegenerative disorders.

#### Studies on neural-immune interactions

We previously reported a marked atrophy of immune organs and alterations in the ratios of  $CD4^+/CD8^+$  and  $CD5^+/CD8^+$  in mice chronically treated with morphine. Using a simple and reliable method for dual-color analysis of heterogenesis cell populations, developed in this laboratory it has been shown that inhibition of calcium influx into splenocytes may be an early event in opiate-induced immunosuppression, and that this action is manifest in  $CD4^+$  but not  $CD8^+$  T cells. Moreover, the action of morphine to suppress immune function appears to be mediated by distinct glucocorticoid dependent and independent mechanisms. The successful infection of primary astrocyte-enriched glial culture with a murine AIDS (MAIDS) virus should facilitate both

characterization of the immunopathology of CNS infection with this virus and serve as a model to evaluate the therapeutic potential of antiviral agents.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 58,501-04 LNS

PERIOD COVERED

October 1, 1989 - September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Receptors for neurotransmitters and drugs in brain and peripheral tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: P. Skolnick, Chief, LN, NIDDK Others: P.K. Arora, Expert;  
A.S. Basile, Senior Staff Fellow; K. Boje, Guest Worker; G.E.  
Evoniuk, Guest Worker; E. Fride, Visiting Fellow; R.T. McCabe,  
Guest Worker; T.D. McIntyre, Staff Fellow; G. Novak, Visiting  
Fellow; I.A. Paul, Guest Worker; J. Schoenheimer, Guest Worker;  
Y. Sei, Visiting Fellow; R.O. Trullas, Visiting Associate; All  
LN, NIDDK.

COOPERATING UNITS (If ~~any~~) A. Jones, DDB, NIDDK; K. Rice, B. DaCosta, LMC, NIDDK;  
K. Jacobson, LBC, NIDDK; H. Yeh, LAC, NIDDK; M.B.H. Youdim, G.  
Kuypers, LCMBG, NIDDK; S. Paul, CNB, NIMH, N. Ostrowski, CPB,  
NIMH; K. Yoshimoto, CPB, NIMH; (Continued)

LAB/BRANCH

Laboratory of Neuroscience

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 209892

TOTAL MAN-YEARS:

10.5

PROFESSIONAL:

10

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

High affinity, stereospecific recognition sites (receptors) for neurotransmitters, neuromodulators, and many clinically useful drugs have been identified in both peripheral tissues and the central nervous system. The interaction of a neurotransmitter, neuromodulator or drug with a specific recognition site initiates a series of events (for example, the opening of an ion channel or activation of an enzyme) resulting in either a physiological response (in the case of a neurotransmitter or neuromodulator) or a pharmacological effect (in the case of a drug). Furthermore, the presence of recognition sites for synthetic substances indicates that endogenous substances may also be present which can mimic (or antagonize) the effects of exogenously applied (synthetic) compounds. Studies are in progress to characterize "recognition-effector" systems, to link novel recognition sites to effector systems, to develop appropriate model systems and methodologies to study these systems, and to relate these systems to both physiological and pathological processes.

ANNUAL REPORT OF THE MOLECULAR PATHOPHYSIOLOGY BRANCH  
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate normal and abnormal cell function at the molecular level with emphasis on transmembrane signalling by hormones, neurotransmitters, growth factors, and other first messengers acting at the cell surface. Approaches used range from molecular biologic techniques to clinical investigation in an effort to define the pathogenesis of diseases characterized by abnormal signal transduction.

Guanine nucleotide binding proteins (G-proteins) as receptor-effector couplers

A family of G-proteins functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for polypeptide hormones, monoamine neurotransmitters, photons of light, chemical odorants, chemotactic factors, and certain growth factors. Effector functions regulated by G-proteins include cAMP formation, cGMP degradation, phosphoinositide breakdown, and several types of ion channel. Major areas of interest concerning G-proteins include: 1) definition of the diversity within this gene family; tissue and subcellular distribution; regulation of gene expression. 2) definition of domains on individual G-protein subunits involved in association of the subunits, attachment to cell membranes, interaction with receptor and effector domains, and possible interactions with other regulatory proteins. 3) definition of the degree and mechanism of specificity for individual G-proteins in coupling to both receptors and effectors. 4) definition of quantitative and qualitative alterations in G-proteins that result in altered signal transduction. Significant recent progress has been made in each of these areas:

1) Molecular basis for subunit association and membrane attachment of GTP-binding proteins- G-proteins are heterotrimers; alpha subunits reversibly associate with a beta/gamma complex. The holoprotein is associated with the cytoplasmic side of the plasma membrane, but the basis for membrane attachment and the domains responsible for subunit association have not been defined. It has been postulated that the beta/gamma complex anchors alpha subunits to the membrane. We have expressed alpha subunits in cos cells and find that the proteins are targeted to the cell membrane, even though alpha subunits can be shown to be expressed in great excess (>10:1) relative to beta subunits. Thus beta subunits cannot be the sole factor responsible for alpha attachment to the plasma membrane. We have found that alpha subunits are attached to the plasma membrane via a 1-2kDa amino-terminal "stalk." By metabolic labeling of transfected cells and immunoprecipitation of expressed alpha subunits, we can show that Gi but not Gs-alpha subunits undergo a specific fatty acid acylation (myristylation). Site-directed mutagenesis of the myristylation site has been performed. The resultant alpha subunit, upon transfection in cos cells, remains in the cytosol, unlike the wild-type which is targeted to the cell membrane. This highlights the critical role of this covalent modification in membrane attachment. The basis for membrane association of the beta/gamma

complex has also been defined. The gamma subunit ends in a "C-A-A-X" motif and has been shown to undergo postranslational processing similar to that of the ras oncogene product, i.e. proteolytic cleavage of the last three amino acids, carboxymethylation of the resultant cysteine, and lipid attachment (likely an isoprenoid moiety) via the cysteine sulphydryl. Elucidation of this pathway has important implications, since inhibition of this processing would preclude attachment of the beta/gamma complex. Under native conditions, beta and gamma subunits are tightly but noncovalently associated. We now find that expression of either beta or gamma subunits in cos cells is dependent on cotransfection of both cDNAs. This implies that a beta/gamma complex is formed early after translation and is necessary for expression of these subunits. [Drs. Jones, Goldsmith, Simonds, Collins, and Spiegel, in collaboration with Dr. Backlund]

2) Altered G-proteins as a cause of altered signal transduction- As critical intermediates in the signal transduction pathway, quantitative or qualitative alterations in G-proteins could have a major impact on the signalling process. We have generated mutant alpha subunits for several G proteins that should lead to either constitutive activation or dominant inhibition of G protein function. The mutant alpha subunits have been initially characterized after transient expression in cos cells. Subsequently stable transfection is being performed to assess the impact of expression of such mutant alpha subunits on cell growth and function. In addition to deliberate creation of mutant G proteins, we are searching for such mutations in a variety of tissues and diseases, including benign and malignant tumors. Using techniques that have been successful in defining mutations in the Gs-alpha gene in pseudohypoparathyroidism (see separate report), we have preliminary evidence for Gi2-alpha gene mutations in adrenal cortical malignancies. [Drs. Weinstein, Merendino, Hermouet, Friedman, and Spiegel]

#### Pseudohypoparathyroidism (PHP)

PHP is a genetic disorder in which resistance to parathyroid hormone (PTH) may be associated with somatic abnormalities collectively termed Albright's hereditary osteodystrophy (AHO). We have previously shown that subjects with this form of PHP are resistant to multiple hormones that act by stimulating cAMP formation, that an approximate 50% reduction in activity of the G-protein (Gs) that couples receptors to stimulation of adenylyl cyclase is present in all tissues from affected subjects, and that subjects with PHP show reduction in steady state mRNA for the Gs-alpha subunit. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects. This work indicates that mutations in a G protein gene can lead to clinically significant disease.

## Molecular biologic studies on the cause of parathyroid neoplasia

Parathyroid tumors (benign adenomas, hyperplasia, and carcinoma) are presumptively due to acquired (and in some cases such as MEN type I to inherited) abnormalities at the gene level. We have begun to study the molecular basis for parathyroid neoplasia by searching for rearrangements and/or deletions in genomic DNA from parathyroid tumors. We have found rearrangement of the parathyroid hormone gene in only 1 of 43 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In contrast, point mutations in ras oncogenes were not found in any parathyroid tumors. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus on 11q13 may encode a tumor "suppressor" gene.

## Honors and Awards:

Lee Weinstein (Senior Staff Fellow) received a Young Investigator Award from the American Society for Bone and Mineral Research for his work on identification of G protein mutations in patients with pseudohypoparathyroidism.

Teresa Jones (Medical Staff Fellow) was invited to present her work on myristoylation of G protein alpha subunits at a Symposium on Membrane Proteins held at State University of New York in Buffalo.

Allen Spiegel (Branch Chief) was invited to lecture at National and International Meetings including the Annual Meeting of the Society of General Physiologists, the Annual Meeting of the British Biochemical Society, a UCLA Conference on G proteins, a Rockefeller University Faculty Lecture, the Endocrine Society Annual Meeting, the 2nd International Congress on Neuroendocrinology, and the European Cell Biology Congress. He also received the Meritorious Service Medal of the U.S. Public Health Service, and was elected to the Association of American Physicians.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59000-03 MPB

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biologic studies on the cause of parathyroid neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK

Others: E. Friedman, M.D., Visiting Fellow, MPB, NIDDK

## COOPERATING UNITS (if any)

S. Marx, M.D.

Chief, Mineral Metabolism Section, MDB, NIDDK

G. Aurbach, M.D.

Chief, MDB, NIDDK

J. Norton, M.D.,

Chief, Surg. Metab. Section, Surgery Branch, NIDDK

## LAB/BRANCH

Molecular Pathophysiology Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary hyperparathyroidism (HPT) is a common endocrine disorder that can cause significant morbidity. HPT may be due to benign neoplasia of a single parathyroid gland (adenoma), benign neoplasia involving multiple parathyroid glands (hyperplasia), and rarely, to malignant neoplasia of a parathyroid gland (carcinoma). The etiology of parathyroid neoplasia has not been defined, but clinical and epidemiologic studies indicate that hyperplasia is often due to an inherited defect (multiple endocrine neoplasia types 1 and 2), and that a history of head and neck irradiation is associated with a significantly higher risk of developing parathyroid neoplasia. As with other forms of neoplasia, parathyroid tumors are presumably due to inherited (germ-line mutation) and/or acquired (somatic mutation) defects in specific genes. Etiologic genetic defects could include inappropriate expression of transforming "oncogenes" and/or loss of expression of tumor "suppressor" genes. The availability of surgically resected parathyroid tumors allows us to search for tumor-specific genetic abnormalities that may be involved in development of parathyroid neoplasia. We have found rearrangement of the parathyroid hormone gene in only 1 of 43 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In contrast, point mutations in ras oncogenes were not found in any parathyroid tumors. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus in 11q13 may encode a tumor "suppressor" gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 59001-25 MPB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Guanine nucleotide binding proteins as receptor-effector couplers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK

Others: T. Jones, M.D., Medical Staff Fellow, MPB, NIDDK

P. Goldsmith, Ph.D., Research Biologist, MPB, NIDDK

W. Simonds, M.D., Senior Staff Fellow, MPB, NIDDK

L. Weinstein, M.D., Medical Staff Fellow, MPB, NIDDK

J. Merendino, M.D., Medical Staff Fellow, MPB, NIDDK

S. Hermouet, M.D., Visiting Fellow, MPB, NIDDK

E. Friedman, M.D., Visiting Fellow, MPB, NIDDK

COOPERATING UNITS (if any)

G. Milligan, Glasgow Univ., Scotland; M. Brann (NINCDS); C. Unson, Rockefeller Univ., N.Y.; P. Backlund (NIMH); M. Forte, Vollum Inst., Oregon

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

11.5

PROFESSIONAL:

8

OTHER:

3.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A family of guanine nucleotide binding proteins (G-proteins) functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for hormones, neurotransmitters, light, odorants, and certain growth factors. Effector functions regulated (positively and, in some instances, negatively) by G-proteins include cAMP formation, phosphoinositide breakdown, potassium and calcium channels, and cGMP degradation. We have used a variety of techniques to study the expression, distribution, regulation, structure and function of G-proteins. Our studies highlight the diversity within the G-protein family. Using peptide specific antibodies, we have defined the specificity of G-proteins in coupling to receptors and effectors. We have defined distinct post-translational lipid modifications necessary for membrane attachment of G protein alpha and beta/gamma subunits. We have created mutations in alpha subunits that cause constitutive activation, and transfected these into cells to define phenotypic effects on cellular function. These studies provide the basis for understanding the role of G-proteins in normal signal transduction and for elucidating possible defects in G-protein structure or function as the basis for abnormal signal transduction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 59002-25 MPB

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on pseudohypoparathyroidism and related disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK  
Others: L. Weinstein, M.D., Medical Staff Fellow, MPB, NIDDK  
E. Friedman, M.D., Visiting Fellow, MPB, NIDDK

COOPERATING UNITS (if any)

P. Gejman, Clin. Neurogenetics Branch, NIMH

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism" (PHP). Patients with this disorder show characteristic constitutional features (Albright's hereditary osteodystrophy - AHO) and do not respond to exogenous parathyroid hormone (PTH). In PHP, UAMP (urinary cyclic AMP) does not increase normally in response to PTH administration. This indicates that there is a defective hormone receptor-adenylate cyclase complex in this disorder. We have shown that many patients with PHP+AHO (PHP Ia) show an approximately 50% reduction in activity of Gs (the stimulatory guanine nucleotide binding protein associated with adenylyl cyclase) in membranes from multiple tissues. Gs deficiency presumably accounts for resistance to multiple hormones in such patients. Using cloned human cDNA probes for the alpha subunit of Gs, we have shown that steady state mRNA levels from fibroblasts of subjects with PHP Ia are reduced by approximately 50% compared with normals. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects.

## **ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY, NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES**

The major research direction of the Laboratory is the elucidation of the structure and function of neurotransmitter systems in the mammalian central nervous system (CNS) and the molecular mechanism of action of CNS active drugs. Also under investigation are peripheral signaling systems and the mechanisms through which the immune and other peripheral systems are influenced by the CNS in normal and disease states. Organic/medicinal chemistry is the foundation of the multidisciplinary approach utilized in these studies which requires synthesis of novel agonists, antagonists, imaging agents, affinity ligands and other drugs for particular applications.

Present work in this Laboratory is concerned with rational design and the synthesis of new, highly selective ligands for drug receptors, using all of the contemporary tools of medicinal chemistry, including computer assisted molecular modeling. Areas now under intense investigation include: (1) central opioid receptor subtypes and peripheral opioid receptors, (2) binding sites on components of the immune system which resemble central opioid receptor subtypes, (3) the mechanism of cocaine and narcotic tolerance and dependence (4) phencyclidine (PCP) recognition sites, (5) sigma, cannabinoid (marijuana) and central and peripheral benzodiazepine receptors and (6) development of new ligands for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging of drug receptors in the CNS of living animals and conscious humans. The multidisciplinary nature of this program requires extensive collaboration with other groups from within and outside of NIH for the purpose of discernment of the structure and function of these receptors and to ensure the practical utility of the discovered ligands provided to biological and biochemical researchers. This Laboratory now is involved in collaborative work with, among others, researchers at Brown University, the University of Alabama, the Medical College of Virginia, the University of Michigan, the Free University in Holland, the University of California (Los Angeles), Meijo University in Japan, the Nathan S. Kline Institute for Psychiatric Research in New York, the Naval Research Laboratory, the Walter Reed Army Institute of Research, the National Institute of Mental Health (ADAMHA), the Nuclear Medicine Department of the Warren Grant Magnuson Clinical Center of NIH, the National Heart, Lung and Blood Institute of NIH, the National Institute of Neurological Disorders and Stroke of NIH, G. D. Searle and Co. and the Laboratory of Neuroscience of NIDDK.

The following summary describes selected advances made by the Laboratory during 1989-1990.

### **Opioid Receptors.**

The unequivocal identification of saturable, enantioselective, high affinity mu, delta and kappa opioid receptor subtypes in the mammalian central nervous system (CNS) has resulted from many converging lines of chemical, pharmacological and biochemical investigation. In addition, evidence continues to accumulate supporting the existence of similar receptor subtypes on components of the immune system. The structure of these subtypes and their function in modulation of the CNS and immune system are extremely important questions and are currently the subject of intense investigation in many laboratories. The significance of such studies has recently been heightened by concerns regarding the relationship of opiate induced immunosuppression to the spread of acquired immune deficiency syndrome (AIDS) in intravenous narcotic abusers. Central to the success of our past and ongoing studies of receptor subtype structure and function has been the synthesis and identification of (a) selective high affinity ligands for each subtype (b) irreversible ligands with receptor subtype specificity and (c) enantiomeric pairs of these ligands for detection of receptor mediated effects.



**Interaction of Endogenous Opioid Peptides and other Drugs with Four Kappa Opioid Binding Sites in Guinea Pig Brain.** Guinea pig brain membranes depleted of mu and delta receptors by pretreatment with the site directed acylating agents, 2-(4-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidazole.HCl (BIT) and N-phenyl-N-[1-(2-(4-isothiocyanato)-phenethyl)-4-piperidinyl]propanamide.HCl (FIT) were used in this study to test the hypothesis that guinea pig brain possesses subtypes of kappa receptors. Pretreatment of membranes with either (-)-(1S,2S)-U50,488 or the kappa selective acylating agent, (1S,2S)-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide, caused a wash-resistant inhibition of kappa-1 binding sites labeled by [3H]U69,593 binding, but not kappa-2 binding sites labeled by [3H]bremazocine. Binding surface analysis of [3H]bremazocine binding resolved two binding sites, termed kappa-2a and kappa-2b, present at densities of 212 and 225 fmol/mg protein, which had low affinity for (-)-(1S,2S)-U50,488 and U69,593. The kappa-2b site had high affinity for beta-endorphin(1-31) ( $K_d=5.5$  nM) and [D-Ala2,D-Leu5]enkephalin ( $K_d=14$  nM), and lower affinity for [D-Ala2-MePhe4,Gly-oI5]enkephalin ( $K_d=147$  nM) and leu5-enkephalin ( $K_d=46.0$  nM). Binding surface analysis of [3H]U69,593 binding also resolved two binding sites, termed kappa-1a and kappa-1b, present at densities of 6.0 and 40.0 fmol/mg protein. The kappa-1a binding site was characterized by very high affinity for alpha-neoendorphin. Quantitative autoradiographic studies demonstrated that kappa-2a and kappa-2b binding sites are heterogeneously distributed in guinea pig brain, and that the anatomical distribution of kappa-1 binding sites reported in the literature is different from that observed in this study for the kappa-2 binding sites. Viewed collectively, these data provide evidence for four kappa receptor subtypes in guinea pig brain.

**beta-FNA Fails to Prevent Naltrexone-induced Upregulation of Mu Opioid Receptors.** This study examined the effect of beta-funaltrexamine (beta-FNA), an irreversible mu receptor antagonist, on naltrexone-induced upregulation of mu (mu-cx + mu-ncx) and delta-ncx opioid receptors. [The subscripts 'cx' and 'ncx' denote binding sites 'in' (cx) and 'not in' (ncx) the opioid receptor complex.] Rats were treated according to the following protocol. Two naltrexone or two placebo pellets were implanted subcutaneously in a nylon mesh on day #1, and were removed intact on day #8. Rats were administered either saline or 20 nmol of beta-FNA in 10 ul of saline (i.c.v.) on day #1,3, 5 and 6, 60 min prior to pellet implantation. Day #9: frozen lysed-P2 membranes were prepared for assay of mu binding sites. In other experiments, membranes were depleted of mu receptors by pretreatment with the site-directed acylating agent 2-(4-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidazole.HCl (BIT) for assay of delta-ncx binding sites using [3H][D-al2,D-leu5]enkephalin. The results demonstrated that beta-FNA did not upregulate mu binding sites, and also did not prevent naltrexone induced upregulation of mu binding sites. Both beta-FNA and naltrexone increased the  $B_{max}$  of delta-ncx binding sites, and their effects were additive. These data suggest that the mechanism(s) responsible for antagonist-induced upregulation of opioid receptors are more complex than previously appreciated.

**Synthesis and Evaluation of a Series of U50,488 Related Isothiocyanate Derivatives as Opioid Receptor Affinity Ligands.** A series of U50,488 related isothiocyanates was synthesized from enantiomerically pure (S,S)-(+)-trans-2-pyrrolidinyl-N-methylcyclohexylamine {(+)-1} and (R,R)-(-)-trans-2-pyrrolidinyl-N-methylcyclohexylamine {(-)-1}. DCC coupling of (+) and (-)-1 with nitrophenylacetic acids followed by catalytic hydrogenation and treatment with thiophosgene afforded a series of six isomeric aryl isothiocyanate analogues of U50,488. Similarly, DCC coupling of (+)- and (-)-1 with (+)- and (-)-N-t-Boc-protected phenylglycines afforded four isomeric alkyl isothiocyanates. Evaluation of the isothiocyanates for their capacity to produce wash-resistant inhibition of mu, delta, and kappa sites *in vitro* was performed using rat and guinea pig brain membranes. None of the compounds was able to irreversibly inhibit binding of [3H]bremazocine to guinea pig and rat brain membranes (depleted of functional mu and delta receptors by pretreatment with acylating agents BIT and FIT).

However, (1S,2S)-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide [(-)-2] was able to specifically and irreversibly inhibit kappa receptors labeled by [3H]-U69,593. Binding analysis revealed the wash-resistant reduction in [3H]-U69,593 binding by (-)-2 to be through an increase in the  $K_d$  without effect on the  $B_{max}$ . (-)-2 failed to effect mu or delta binding in rat or guinea pig brain under the same conditions. The enantiomer of (-)-2, (1R,2R)-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide [(+)-1], failed to affect kappa receptors labeled by [3H]-U69,593 under the same conditions as for (-)-2. (1S,2S)-trans-3-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl]benzeneacetamide [(-)-3] inhibited to 49.6% of the control, in a wash-resistant manner, kappa receptors labeled by [3H]-U69,593. However, (-)-3 was not as selective as (-)-2 since it also reduced [3H]DADLE (delta) binding to 82.4% of the control value. (1S,2S)-trans-4-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl]benzeneacetamide exhibited selective wash-resistant inhibition of delta receptors labeled by [3H]DADLE resulting in a reduction in binding to 42.9% of control. In the alkyl isothiocyanate series, (1S,2S)-trans-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-(S)-2-phenyl-2-isothiocyanatoacetamide [(-)-4] also showed the capacity to selectively inhibit [3H]-U69,593-sensitive kappa sites, resulting in a reduction in binding to 72.2% of control at 1  $\mu$ M while (+)-4 was inactive. None of the amino precursors of the isothiocyanates exhibited the capacity for wash-resistant inhibition of any of the receptor systems tested. Although intracerebroventricular (icv) injection of the most potent compound (-)-2 into guinea pig brain failed to produce any irreversible inhibition of delta receptors, icv injection of the less potent (-)-3 into guinea pig brain resulted in a significant reduction in the kappa receptors that bind [3H]-U69,593, but not those that bind [3H]bremazocine. All of the compounds that showed the capacity to irreversibly inhibit kappa receptors labeled by [3H]-U69,593 in vitro possessed the 1S,2S absolute configuration.

Synthesis of High Specific Activity Tritium Labelled 1S,2S-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, a Specific Irreversible Ligand for Kappa Opioid Receptors. Optically pure tritium labeled 1S,2S-(-)-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, an affinity ligand specific for the kappa opioid receptor was synthesized from optically pure 1S,2S-(-)-trans-2-amino-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide via the sequence of dibromination (57%) followed by catalytic tritiation of the dibromide. The resulting tritium labelled aniline (14% yield, specific activity 31.2 Ci/mmol) was transformed to the title compound in 13.3% yield and 99+% radiochemical purity by treatment with thiophosgene.

Apparent Down Regulation of Mu and Kappa Opioid Binding Sites Following the Chronic Administration of the Potent 5HT Reuptake Blocker Clomipramine. This study examined the effect of chronic clomipramine administration on opioid mu- and kappa-binding sites. Clomipramine or saline was administered to rats via osmotic minipumps. Lysed-P2 brain membranes were prepared and preincubated without (control membranes) or with the mu-selective acylating agent, 2-(4-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanato- benzimidazole-HCl (BIT), to deplete membranes of mu-binding sites. [3H]6-Desoxy-6-beta-fluoronalaltrexone ([3H]cycloFOXY) was used to label mu- and kappa-binding sites. Weighted nonlinear least squares analysis of cycloFOXY binding surfaces permitted determination of the  $K_d$  and  $B_{max}$  values of mu- and kappa-binding sites in control and treated rats. Subacute (3 days) administration of rats with clomipramine had no significant effect on [3H]cycloFOXY binding. Chronic (28 days) administration of clomipramine produced a small (approximately 10%) but statistically significant decrease in the  $B_{max}$ . These findings, and other studies that have examined the effect of chronic antidepressant administration on opioid receptors, indicate that the endogenous opioid systems may play a role in obsessive-compulsive disorder.

Apparent Affinity of Opioid Antagonists in Morphine-Treated Rhesus Monkeys Discriminating between Saline and Naltrexone. In morphine-treated rhesus monkeys discriminating between s.c. injections of naltrexone and saline while responding under a fixed-ratio schedule of stimulus shock termination, the apparent affinity of antagonists for opioid receptors was estimated using two different methods: 1) substitution studies in which cumulative doses of drug were administered to monkeys that received morphine 3 hr before the session and 2) studies in which single doses of antagonists were administered before cumulative doses of agonists in monkeys that had received saline 3 hr before the session. The ED50 values from the substitution study were compared to apparent pA2 values determined when antagonists were administered before agonists. Apparent affinities (pA2) for antagonists, estimated by their capacity to prevent the effects of the opioid agonist alfentanil, were: naltrexone = 8.69, naloxone = 7.78, quadazocine = 7.55, nalorphine = 7.31 and naltrindole = 5.29. The pA2 values were highly correlated ( $r = 0.94$ ) with ED50 values determined in substitution studies. These results demonstrate that substitution by opioid antagonists for the naltrexone discriminative stimulus in morphine-treated monkeys provides a good estimate of affinity for opioid mu receptors as supported by independent pA2 analyses. These data also suggest affinity of opioid antagonists for opioid mu receptors is not changed in monkeys treated daily with morphine and, furthermore, support the notion that binding to opioid receptors is the mechanism by which antagonists precipitate withdrawal in opioid-dependent monkeys.

Alterations in Locomotor Activity and Ingestive Behaviors Following Intraventricular Injections of (S,S) and (R,R) U50,488 (a Kappa Opiate Agonist). The role of the brain kappa receptor in mediating a variety of behavioral effects was assessed by using the (S,S) and (R,R) stereoisomers of the selective kappa agonist U50,488. In all the behavioral tests (S,S) U50,488 was found to be the active enantiomer. (R,R) U50,488 was inactive in tests of ingestion and produced nonspecific depressant effects on locomotor activity which could not be reversed by naloxone. In tests of locomotor activity, (S,S) U50,488 increased locomotor output. In tests of ingestion (S,S) U50,488 increased palatable food intake in nondeprived rats and decreased water intake in 21 hour water-deprived rats. A time-course study, however, showed an increase in water intake four hours following (S,S) U50,488 administration. Drug specificity was further established by the antagonism of (S,S) U50,488-induced increases in locomotor activity and palatable food intake by naloxone at a 5.0 mg/kg and 1.0 mg/kg dose, respectively.

Kinetic Analysis of the Opiate Antagonist Cyclofoxy in Rat Brain. The opiate antagonist (-)-cyclofoxy [(-)-CF] and the receptor inert enantiomer (+)-CF were radiolabeled with 18F or 3H and administered to conscious Sprague Dawley rats; an isotope effect was not observed. Constant i.v. infusion of both 18F-(-)-CF and 3H-(+)-CF in tracer amounts showed a marked difference in the tissue level of 18F-(-)-CF among various brain structures, whereas the values for 3H-(+)-CF were lower and much less variable. Coinfusion of unlabeled (-)-CF did not change the tissue binding of 3H-(+)-CF in any brain structure, but reduced that of 18F-(-)-CF to the same level as 3H-(+)-CF. These results demonstrate an identical nonspecific tissue binding for (+)- and (-)-CF *in vivo*, and suggest that (+)-CF can be used to measure the "nonspecific" component of (-)-CF binding in brain. A nonlinear analysis of the 3H-(+)-CF data indicated the presence of both "instantaneous" and time-dependent components in nonspecific tissue binding and that nonspecific binding varied 1.5-fold in different brain structures. The combined 3H-(+)-CF and 18F-(-)-CF data were fitted to a four-compartment model which includes parameters for capillary transport, "instantaneous" and time-dependent nonspecific tissue binding, as well as receptor association ( $k_3$ ) and dissociation ( $k_4$ ). The rate constant  $k_3$  varied considerably in various structures of cerebrum (2.0 to 8.7 min<sup>-1</sup>), while  $k_4$  was estimated within a narrow range (0.12-0.17 min<sup>-1</sup>). The receptor binding potential ( $k_3/k_4$ ) ranged between 12.7-56.2 and was in good agreement with previous estimates *in vitro*.

Transport and Rapid Tissue Binding of the Opiate Antagonist Cyclofoxy: Comparison of Active and Inactive Enantiomers. The "rapid phase" brain distribution of 3H-labeled enantiomers of the opiate receptor antagonist cyclofoxy (CF), receptor active (-)- and inert (+)- forms, was measured during 20-180 sec i.v. infusion in rats. 14C-Iodoantipyrine was co-infused during these experiments to obtain a simultaneous measure of blood flow. The influx clearance (K1) and the rapid binding equilibrium constant (Keq) were estimated in different brain regions for both enantiomers (two-compartmental model); a possible receptor binding process (k3) was also examined for (-)-CF (three-compartment model). K1 (0.46-0.91 ml/min/g), the capillary permeability-surface area product (PS: 0.75 to 1.4 ml/min/g) and the tissue extraction fraction (E: 0.6 - 0.7) were found to be identical for both enantiomers in the non-receptor binding model; Keq was identical in cerebellum, but larger for (-)-CF in other brain structures. The difference in Keq between the enantiomers (two-compartment model) and the magnitude of k3 for (-)-CF (three-compartment model) correlated with the rank order of opiate receptor density observed in vitro and in vivo. These results suggest that concomitant use of (-)-CF and (+)-CF will be useful for in vivo receptor binding analyses.

Anti-Opioid Receptor Antibody Recognition of a Binding Site on Brain and Leukocyte Opioid Receptors. Opioid receptors reportedly exist on neuronal tissue of central and peripheral origin as well as on cells of the immune system. Previously, an opioid receptor was purified from the neuroblastoma x glioma hybrid cell line, NG 108-15 cells. In an effort to compare these results with opioid receptors isolated from primary neuronal tissue, we employed a methodology based on the molecular recognition theory to develop a monoclonal antibody which was used to isolate and biochemically characterize murine brain opioid receptors. We have now purified an opioid receptor from mouse brain with a molecular weight of 65,000 daltons (range was 62-70 kD under reducing conditions) using a monoclonal antibody to an (the) opioid receptor. In situ labeling experiments with the delta-class selective opioid receptor affinity ligand, cis-(+)-3-methylfentanylisothiocyanate (SUPERFIT) of brain membrane confirmed these observations. Moreover, SUPERFIT, when coupled to the binding site, could block the recognition of the receptor by the monoclonal antibody. However, the selective, mu-class opioid receptor affinity reagent, 2-(p-ethoxybenzyl)-1-N,N-diethylaminoethyl-5-isothiocyanato- benzimidazole was ineffective at masking the binding site from recognition by the monoclonal antibody. Likewise, opioid-like receptors were purified from murine leukocytes which migrated at a molecular weight of 58,000 daltons under nonreducing conditions and 70,000 daltons under reducing conditions. In addition, immunoaffinity-purified receptor is shown to specifically bind the delta-class-selective opioid ligand, cis-(+)-3-methylfentanylisothiocyanate as well as the endogenous opioid peptides, beta-endorphin and [Met]-enkephalin. These results demonstrate that the opioid receptor isolated from brain tissue is structurally and antigenically similar to the delta-class opioid receptor from NG108-15 cells and cells of the immune system.

Corticotropin-releasing Hormone Augments Natural Killer Cell Activity Through a Naloxone-sensitive Pathway. Overnight treatment of murine leukocytes with corticotropin-releasing hormone (CRH) and arginine vasopressin enhances natural killer cell activity. Moreover, the opioid receptor antagonist, naloxone, as well as the delta-class opioid receptor antagonist, naltrindole, can block this effect. The responsiveness of murine leukocytes to CRH is both dose- and time-dependent. The effector cells are both MAC-1 and Thy-1.2 antigen-positive. Whereas, beta-endorphin is also shown to enhance natural killer cell activity in a naloxone-reversible manner, adrenocorticotrophic hormone (ACTH) has a negligible effect. Macrophage depletion prior to incubation with CRH blocks the CRH-induced natural killer cell augmentation. These results suggest hypothalamic-releasing hormones such as CRH may have a biologically relevant role in the modulation of immune cells either directly or indirectly through the induction of neuropeptide hormones known to have immunomodulatory capabilities.

The Committee on Problems of Drug Dependence Drug Testing Program. In vitro and in vivo assays have been used to study new analgesics, and stimulants and depressants, under the auspices of the Committee on Problems of Drug Dependence, with the goal of determining their physical dependence potential and abuse liability. Data from the studies on stimulants and depressants have been utilized by the Expert Committee of the World Health Organization which is charged with the evaluation of scientific data used for drug scheduling under the Psychotropic Substances Convention.

### Studies Towards the Development of a Cocaine Antagonist.

Tight binding dopamine reuptake inhibitors as cocaine antagonists. It was noted that tight binding dopamine (DA) reuptake inhibitors might act as cocaine antagonists. Binding studies demonstrated that the high affinity dopamine reuptake inhibitor, 1-[2-bis(4-fluorophenyl)-methoxy]ethyl]-4-[3-phenylpropyl] piperazine (GBR12909) produced a wash resistant inhibition of the DA transporter in rat striatal membranes as labeled by [ $^3\text{H}$ ]cocaine or [ $^3\text{H}$ ]1-[2-(diphenyl-methoxy)ethyl]-4-(3-phenylpropyl)piperazine ([ $^3\text{H}$ ]GBR12935), indicative of tight binding. In vivo microdialysis experiments showed that administration of 25 mg/kg GBR12909 to rats produced a modest, but not statistically significant, increase in the extracellular levels of striatal DA, while this same dose of GBR12909 inhibited the ability of cocaine to elevate extracellular DA levels by 64%. These data suggest that tight binding DA reuptake blockers may provide a fruitful approach for developing a cocaine antagonist.

### Phencyclidine Recognition Sites.

We have studied the action of phencyclidine (PCP)-like ligands on glutamate receptors of the N-methyl-D-aspartate (NMDA) type. Phencyclidine binding sites have been implicated as allosteric sites which interact with glutamate receptors of the NMDA type. Some phencyclidine (PCP)-like compounds have recently been reported to exert a robust protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. Other sites for interaction with PCP-like ligands in the CNS have also been found, including the dopamine uptake complex.

Metaphit Inhibits Dopamine Transport and Binding of [ $^3\text{H}$ ]Methylphenidate. Metaphit, an acylating derivative of PCP, was shown to interact with components of the dopamine nerve terminal in rat striatal tissue. This compound, previously demonstrated to be an irreversible inhibitor at the phencyclidine receptor, was shown in these experiments to irreversibly inhibit synaptosomal [ $^3\text{H}$ ]dopamine uptake. It also inhibited binding of [ $^3\text{H}$ ]methylphenidate to its recognition site, which is thought to be a subunit of the dopamine transporter. Although the inhibition was due primarily to a reduction in the binding and transport capacity of the systems studied, increases in the apparent  $K_D$  of [ $^3\text{H}$ ]methylphenidate and the  $K_m$  of [ $^3\text{H}$ ]dopamine were also observed. Differences in the behavior of metaphit and phencyclidine in these dopaminergic systems compared to their effects on the NMDA receptor-linked phencyclidine receptor suggest that metaphit may be interacting with two distinct molecular sites in the rat striatum.

Synthesis and Anticonvulsant Activity of 1-Phenylcyclohexylamine Analogues. Thirty-eight analogues of 1-phenylcyclohexylamine (PCA), a PCP derivative, were examined for their activities in the mouse maximal electroshock (MES) seizure test and in a motor-toxicity assay. We determined the binding affinities of the compounds for PCP acceptor sites in rat brain membrane. Many of the analogues were protective against MES seizures and caused motor toxicity. The potencies in the motor toxic seizure tests showed a moderate correlation with the affinities for PCP sites. Several analogues exhibited a greater separation of potencies in the motor toxicity

and MES seizure tests than did the parent compound PCA. These were obtained by (i) 3-methylation of the cyclohexyl ring trans to the phenyl ring, (ii) methoxylation at the ortho position on the phenyl ring, and (iii) contraction of the cyclohexane ring to form the corresponding cyclopentane.

Interactions between Phencyclidine and Nifedipine at 45-Calcium ion-uptake Sites. The effects of PCP and the acylating derivatives fourphit and metaphit were investigated on potassium ion-stimulated 45-calcium ion-uptake into a preparation of mouse brain neurons. PCP produced a concentration dependent inhibition of depolarization-dependent 45-calcium ion-uptake (IC50 4.0  $\mu$ M) which was reversed by washing of the neurons. Treatment of neurons with 100  $\mu$ M, but not 10  $\mu$ M of the PCP acylating drugs fourphit and metaphit followed by washing resulted in a significant inhibition (26%-38%) of depolarization-dependent 45-calcium ion-uptake. Nifedipine (10  $\mu$ M) produced a significant inhibition (33%) of 45-calcium ion-uptake only in neurons treated with 10  $\mu$ M fourphit. These results suggest that PCP can interact with neuronal calcium channels in several ways to alter their function.

Evidence for N-Methyl-D-aspartate-coupled and Dopamine Reuptake Carrier-associated Phencyclidine Binding Sites. Numerous studies have demonstrated that a binding site for the psychotomimetic drug PCP exists within the receptor channel complex for the excitatory amino acid neurotransmitter, glutamate, specifically the glutamate receptor selectively activated by N-methyl-D-aspartate (NMDA). Several lines of evidence support the hypothesis that all PCP receptors in rat brain are associated with the NMDA receptor complex. In this study we reexamined this hypothesis. The PCP analog [3H]1-[1-(2-thienyl)cyclohexyl]piperidine ([3H]TCP) labels two high affinity binding sites in membranes prepared from guinea pig brain: site 1 ( $K_d$ = 14.1 nM,  $B_{max}$ = 631 fmol/mg protein) and site 2 ( $K_d$ =46.5 nM,  $B_{max}$ =829 fmol/mg protein). (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (+)-MK-801 bound to site 1 with high affinity ( $K_i$ =3.2 nM) and to site 2 with low affinity ( $K_i$ =5208 nM). The order of potency of drugs for inhibiting [3H]TCP binding to site 1 correlated with their ED50's for inhibition of NMDA-mediated responses reported in the literature, whereas the order of potency of drugs for inhibiting [3H]TCP binding to site 2 correlated with their ED50's for inhibition of [3H]dopamine reuptake reported in the literature. Kinetic experiments demonstrated that glutamate, 2-amino-7-phosphonoheptanoic acid (AP7), and Mg ion modulated [3H]TCP binding to site 1, but not site 2. Preincubation of guinea pig striatal membranes with varying concentrations of the high affinity dopamine reuptake inhibitors (N-[1-(2-benzo(b)thiophenyl)cyclohexyl]piperidine (BTCP) and 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl] piperazine (GBR12909), caused a wash-resistant inhibition of [3H]TCP binding to site 2, but not site 1. Taken collectively, these data demonstrate the existence of a high affinity PCP binding site associated with the dopamine reuptake carrier, and raise the possibility that the therapeutic and psychotomimetic effects of PCP in humans are separable and mediated via different binding sites.

The Effects of MK-801 on Amnesia. The effects of optical isomers of 5-methyl-10,11-dihydro-5H-dibenzo(a, d)cyclohepten-5,10-imine maleate (MK-801) and PCP on amnesia induced by carbon monoxide (CO) in mice were investigated by using the passive avoidance task method, since these drugs have neuroprotective effects on delayed degeneration induced by ischemia. In the mice exposed to CO before training, at the time of acquisition of a memory and after the consolidation of a memory, acute and delayed amnesia were induced. (+)-MK-801 and (-)-MK-801 improved the delayed amnesia, but the effects of PCP were weak; (+)-MK-801 and PCP potentiated the acute amnesia. From these results, it is suggested that there is a stereoselectivity in the effects of MK-801 on CO-induced amnesia and that CO-induced delayed amnesia animals could be used as an ischemic amnesia model.

**Synthesis and Pharmacological Effects of meta-Substituted Phenylcyclohexyl]-1,2,3,6-tetrahydropyridines.** A series of 1-[1-aryl(cyclohexyl)]-1,2,3,6-tetrahydropyridines were prepared by the reaction between 1-(1-cyanocyclohexyl)-1,2,3,6-tetrahydropyridine and an appropriately substituted Grignard reagent. The resulting compounds were tested for their PCP binding site affinities. Selected compounds were then tested for their ability to produce ketamine appropriate responding in monkeys and/or to show neuroprotective effects in a baby rat hypoxia/ischemia model. Binding site affinity correlated well with discriminative stimulus effects, but was a poor indicator of neuroprotective efficacy within this series.

**Structural and Conformational Aspects of Binding of Aryl-alkyl Amines.** The structural requirements for binding to the PCP site with the MK-801 class of compounds was examined. The polycyclic nature of MK-801 was analyzed as a set of substructures, including both the phenyl and amino groups. It was found that the structural and conformational requirements for binding were strict within this class of compounds; analogues with free rotation of one of the phenyl groups showed greatly diminished receptor affinities. Thus, MK-801 was best viewed as a conformationally restricted 1,2-diphenylethylamine derivative.

**Synthesis and Structure-Activity Relationship of C5-Substituted Analogues of Racemic-Desmethy-MK-801.** A series of eight C5-substituted analogues of (+)-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imines have been prepared by the directed lithiation-alkylation (and acylation) of its (+)-N-tert-butylformamidinyl derivative followed by formamidine solvolysis. An additional 10 analogues were prepared by elaboration of the C5-ethyl ester derivative. Analogues possessing large (e.g. propyl and larger) lipophilic substituents displace [3H]TCP from the high-affinity PCP binding site in rat brain homogenates only at high concentrations ( $K_i$  greater than 1000 nM); however, the presence of a polar amino functionality (e.g. 2-aminoethyl) offsets this effect ( $K_i = 20$  nM). Thus, the boundary condition for lipophilic substituents larger than ethyl appears to be polar in nature. Interaction of the 11 relatively small ( $MR$  less than 14) C5-substituted analogues of 1 with the high-affinity PCP binding site associated with the N-methyl-D-aspartate (NMDA) receptor is best described by the equation  $\log (1/K_i) = 5.83F + 0.641\pi + 7.41$  ( $r = 0.90$ )).

**Specificity of Phencyclidine-like Drugs and Benzomorphan Opiates for two High Affinity Phencyclidine Binding Sites in Guinea Pig Brain.** Recently, we described the presence of two high affinity PCP binding sites in guinea pig brain, with one site coupled to the glutamate excitatory amino acid receptor specifically activated by N-methyl-D-aspartate (NMDA) (site 1) and the other site associated with the dopamine (DA) reuptake carrier (site 2). PCP and its analogs, as well as the benzomorphan opiates, are known to interact with PCP binding sites. We determined the equilibrium dissociation constants ( $K_d$ ) of these compounds for the two PCP binding sites. PCP and 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP), an analog of PCP, were essentially non-selective between the two sites and also were the two drugs of the group observed to have the highest affinity for site 2. (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine [(+)-MK-801] was the most selective agent for site 1; none of the drugs tested showed selectivity for site 2. In humans, PCP produces psychotomimetic effects, while (+)-MK-801 has been reported to produce minimal, if any, psychotomimetic effects at doses sufficient to reduce seizures. These clinical observations in conjunction with the present biochemical binding data suggest that (+)-MK-801 may serve as a "marker" for site 1 and that the psychotomimetic effects of PCP might be mediated by site 2.

**Inhibition of Potassium-evoked Release of Cholecystokinin.** Potassium-evoked release of cholecystokinin (CCK) from slices of caudate-putamen, hippocampus, and cerebral cortex was inhibited in a dose-related fashion by PCP. In order to further examine this effect, PCP-like ligands, dexoxadrol, levoxadrol, PCMP and MK-801 as well as compounds known to interact with the sigma receptor ((+)-SKF, DTG, (+)-3PPP, and pentazocine) were tested. While some of

these compounds inhibited CCK release, their rank order of potency (Dex = Lev, PCP = PCMP, DTG = MK-801 = (+)-3PPP) differs from that of known PCP-NMDA linked effects or sigma interactions. These results suggest that the mechanism by which PCP acts to inhibit CCK release may involve a novel type of PCP interaction.

## **Cannabinoid Receptors.**

**Cannabinoid Receptor Localization in Brain.** [3H]CP 55,940, a radiolabeled synthetic cannabinoid, which is 10-100 times more potent in vivo than delta9-tetrahydrocannabinol, was used to characterize and localize a specific cannabinoid receptor in brain sections. The potencies of a series of natural and synthetic cannabinoids as competitors of [3H]CP 55,940 binding correlated closely with their relative potencies in several biological assays, suggesting that the receptor characterized in our in vitro assay is the same receptor that mediates behavioral and pharmacological effects of cannabinoids, including human subjective experience. Autoradiography of cannabinoid receptors in brain sections from several mammalian species, including human, reveals a unique and conserved distribution; binding is most dense in outflow nuclei of the basal ganglia—the substantia nigra pars reticulata and globus pallidus—and in the hippocampus and cerebellum. Generally high densities in forebrain and cerebellum implicate roles for cannabinoids in cognition and movement. Sparse densities in lower brainstem areas controlling cardiovascular and respiratory functions may explain why high doses of delta9-tetrahydrocannabinol are not lethal.

## **Sigma Receptor Ligands.**

Sigma receptors are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs, as well as some of the (+)-enantiomers of the benzomorphan opioids. Studies utilizing various sigma ligands have implicated sigma receptors in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. Although progress is being made in elucidating the physiological roles of sigma receptors, the biochemical systems modulated by sigma receptors are not clear.

**Synthesis and Evaluation of a New Class of Highly Potent and Selective Sigma Receptor Probes.** Certain benzeneacetamides [(+)- and (-)-cis-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide] were recently reported to be potent sigma receptor ligands. In order to determine whether efficacy for the sigma receptor could be improved, a series of compounds related to the benzeneacetamides, N-substituted cis-2-(1-pyrrolidinyl)-N-methylcyclohexylamines, were synthesized and their SAR were determined. The compounds were synthesized starting with the previously reported racemic-, 1S,2R-(+)-, and 1R,2S-(-)-cis-2-(1-pyrrolidinyl)-N-methylcyclohexylamines. Analysis of sigma ([3H](+)-PPP), kappa ([3H]bremazocine and [3H]U69,593), dopamine-D2 ([3H]sulpiride) and phencyclidine (PCP) ([3H]TCP) receptor binding in guinea pig brain revealed a number of highly potent and selective sigma receptor ligands. Notably, 1S,2R-cis-(-)-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-2-(2-naphthyl)acetamide (1) ( $K_i=8.7$  nM), racemic-cis-2-amino-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide ( $K_i=11$  nM), 1S,2R-(-)-cis-N-methyl-N-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine ( $K_i=1.3$  nM) and 1R,2S-(+)-cis-N-methyl-N-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)-cyclohexylamine (2) ( $K_i=6$  nM) exhibited very high affinity at sigma receptors, by displacement of [3H](+)-3-(3-hydroxyphenyl)-N-(1-propyl)-piperidine ([3H](+)-3-PPP). These compounds showed insignificant affinity for kappa, dopamine or PCP receptors, making them valuable tools for the study of sigma receptors. Furthermore, these compounds also exhibited enantioselectivity ranging from five-fold for (+)- and (-)-2 to 160-fold for (+)-



and (-)-1. Several other compounds showed equivalent selectivity but displayed lower sigma receptor affinity. Analysis of structure versus affinity for these sigma ligands revealed that compounds derived from a pharmacophore containing an aromatic ring (with hydrophobic groups in the 3- and 4- positions) coupled to the cis-2-(1-pyrrolidinyl)-N-methylcyclohexylamine moiety via a lipophilic sidechain would afford potent and selective sigma ligands. Generally, the arylacetamides showed higher kappa receptor affinity than the 2-arylethylamines with both groups of compounds being more selective at the sigma receptor. The arylethylamine 1S,2R-(-)-cis-N-methyl-N-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine [(-)-2] proved to be the most potent and selective known sigma receptor ligand. This compound represents the first member of a novel class of highly potent and selective sigma receptor probes which will be important for further evaluation of the structure and function of this receptor. In behavioral tests, arylethylamine 1S,2R-(-)-2 ( $K_i=1.3$  nM, [3H](+)-3-PPP) failed to produce any significant effects up to 100 nmol/rat administered intracerebroventricularly (icv). In contrast, DTG ( $K_i=49$  nM, [3H](+)-3-PPP) potentially produced stereotyped behavior ( $ED_{50}=55$  nmol/rat) and ataxia ( $ED_{50}=57$  nmol/rat). Racemic arylacetamide 1 did not produce any significant stereotyped behavior or ataxia in rats when administered at 10 mg/kg interperitoneally (ip) or 18 mg/kg subcutaneously (sc). However, racemic 1 produced stereotyped behavior and ataxia after icv administration, but less potently than DTG.

**Sigma Receptors and Signal Transduction.** Attenuation of signalling via the phosphoinositide pathway is a potential mechanism by which sigma ligands could modulate the functions of neurotransmitters in vivo. Sigma receptors appear to be one of a growing number of receptors postulated to utilize a variety of mechanisms to effect negative modulation of phosphoinositide turnover. Further studies will be aimed at the in vivo interactions of neurotransmitters and sigma ligands as well as further elucidation of the mechanism of the sigma effect. Furthermore, inhibition of the cholinergic phosphoinositide response provides a convenient bioassay system for use in development and characterization of novel sigma agonists and antagonists.

**Drug Specificity of Pharmacological Dystonia.** Three (+)-benzomorphans with significant binding affinity for sigma receptors ( $K_i=35-530$  nM) produced dystonia in a dose-related manner when microinjected into the red nucleus of rats. These effects appear to be related to the sigma-binding properties of the compounds since similar injections of non-sigma ligands were without effect. (+)-Nordihydrocodeinone, a structurally related (+)-opiate with negligible affinity for sigma receptors, failed to significantly alter the normal posture of rats. Intrarubral injections of various vehicles and selective dopaminergic and serotonergic compounds were also without effect. Likewise, (+)-[3-(3-hydroxyphenyl)-N-(1-propyl)-piperidine] ((+)-3PPP), a ligand with mixed activity at sigma and dopamine receptors, was without effect. Although the combination of high affinity for a receptor and the failure to produce a physiological response sometimes reflects the actions of an antagonist, (+)-3PPP does not appear to be a sigma receptor antagonist since it failed to attenuate the dystonia produced by another sigma compound.

**Autoradiographic Distribution of [3H](+)-Pentazocine Binding Sites in Guinea Pig Brain.** The distribution of [3H](+)-pentazocine binding sites in the guinea pig brain was examined using autoradiography. [3H](+)-Pentazocine binding was high in the cingulate cortex, dorsal diagonal band, periaqueductal gray, cerebellum and cranial nerve nuclei. A high correlation was found between the binding of [3H](+)-pentazocine and [3H]-1,3-di-o-tolylguanidine (DTG), a selective sigma ligand.

**Characterization of [3H](+)-Pentazocine, a Highly Selective Sigma Ligand.** Competition binding studies using [3H](+)-3-PPP and [3H]TCP in guinea pig brain have shown that unlabeled (+)-pentazocine exhibits 5,600-fold selectivity for sigma receptors over PCP receptors compared to a 4-fold selectivity for unlabeled (+)-SKF 10,047. Therefore, in addition

to its higher binding affinity at sigma receptors, [3H](+)-pentazocine is a much more selective benzomorphan-based probe than [3H]-(+)-SKF 10,047. The availability of [3H](+)-pentazocine should greatly facilitate studies of sigma receptor sites. The only other sigma ligand of comparable affinity is [3H]haloperidol ( $K_d = 2.0$  nM). However this ligand has very limited utility as a sigma receptor probe due to its very high affinity for dopamine receptors. Also of interest is our observation that [3H]-(+)-pentazocine exhibits greater than 95% specific binding even at relatively high concentrations.

#### **1989-1990 Non-project Activity.**

Dr. Kenner C. Rice continues to serve as a member of the Editorial Advisory Board of the Journal of Medicinal Chemistry and as an elected member of the Executive Committee of the Organic Chemistry Division of the American Chemical Society (ACS). In the latter capacity, he serves as liaison with the Biotechnology Secretariat of the ACS. He continues as an elected member of the Board of the Committee of Problems on Drug Dependence (CPDD) representing the American Chemical Society and also as Chairman of the CPDD Immune Function Testing Committee. Dr. Rice served as a member of Peer Review Panel C for the Walter Reed Army Institute of Research Promotion Board. He continues to serve on the selection committee for national and international research awards.

Dr. Arthur E. Jacobson was reappointed as Chairman of the Animal Testing Program of the Committee on Problems of Drug Dependence for 1990-1991, and as Affiliate Professor in the Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University. He has been awarded the J. Michael Morrison award, a biennial award of the Committee on Problems of Drug Dependence for outstanding contributions in the area of scientific administration related to drugs of abuse. He continues to serve on the selection committee for the international Sato Memorial award, and is on the Research Evaluation Panel for the Walter Reed Army Institute. He has been invited to serve as Chairman and to lecture at the International Symposium on Drug Dependence in Mexico City sponsored by the Escuela Militar de Graduados de Sanidad.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 59,501-04LMC

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Design and Synthesis of Drugs Acting on Central and Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. C. Rice, Laboratory Chief, LMC-NIDDK

Co PI: A. E. Jacobson, Research Chemist, LMC-NIDDK

OTHERS: A. Reid, Biochemist, LMC-NIDDK; B.R. de Costa, Visiting Associate, LMC-NIDDK; N. A. Grayson, IRTA, LMC-NIDDK; M. Seggel, IRTA, LMC-NIDDK; R. J. Weber, Staff Fellow, LMC-NIDDK; M. Mattson, Biologist, LMC-NIDDK; L. Band, IRTA, LMC-NIDDK; W. Williams, Microbiologist, LMC-NIDDK; H. Xu, Visiting Associate, LMC-NIDDK; R. B. Rothman, Sr. Research Investigator, LMC-NIDDK; H. Akkunne, Fogarty Fellow, LMC-NIDDK; L. Radesca, LMC-NIDDK.

COOPERATING UNITS (if any)

WRAIR (F. Tortella); LN-NIDDK (P. Skolnick); U of Michigan (J. Woods, C. P. France); Free U., Holland (A. Schoffeleer); CC-NM (D. Kiesewetter, M. Channing); U of Alabama (E. Blalock); BPB-NIMH (A. Pert); Medical College of Virginia (E. L. May, L. S. Harris, M. Aceto).

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

9.7

PROFESSIONAL:

8.7

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The primary goals of this program are to increase understanding of the structure and function of neurotransmitter systems in the overall operation of the mammalian central nervous system (CNS), and the molecular mechanism of action of drugs which act on the CNS, as well as the mechanism(s) through which the immune and other peripheral systems are influenced by the CNS in normal and disease states. The multidisciplinary approach utilized in this program employs rational drug design based on structure-activity relations and molecular modeling, modern organic chemical synthesis, pharmacology, biochemistry, immunology and requires collaboration with other appropriate disciplines. Elucidation of the molecular structure and mechanism of action of the ligand-receptor systems and their antagonists provides new opportunities for the design of superior drugs and insight into disorders which are now little-understood. Synthetic programs are continuing to develop new ligands for imaging brain drug receptors by positron emission tomography (PET) and single photon emission computed tomography (SPECT) scanning.

Our data provide evidence for four kappa opioid receptor subtypes in guinea pig brain. Quantitative autoradiographic studies demonstrated that two of these kappa sites are heterogeneously distributed, and that their anatomical distribution is different than those previously reported. We have synthesized optically pure tritium labeled 1S,2S-(-)-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, an affinity ligand specific for the kappa receptor.

We have developed a monoclonal antibody to isolate and biochemically characterize brain opioid receptors. An opioid receptor from mouse brain was purified with a molecular weight of 65,000 daltons. The opioid receptor isolated from brain tissue was found to be structurally and antigenically similar to the delta-class opioid receptor from NG108-15 cells and cells of the immune system.

The NIH Opiate Total Synthesis continues to be employed to provide previously inaccessible unnatural enantiomers of opiates and derivatives as new pharmacological agents and research tools.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 through September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Design, Synthesis and Evaluation of Medicinal Agents and Research Tools

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. E. Jacobson, Research Chemist, LMC-NIDDK

Co PI: K. C. Rice, Laboratory Chief, LMC-NIDDK

## OTHERS:

A. Reid, Biochemist, LMC-NIDDK; B. R. de Costa, Visiting Associate, LMC-NIDDK; P. Hillery, Special Volunteer, LMC-NIDDK; M. Mattson, Biologist, LMC-NIDDK; H. Akunne, Fogarty Fellow, LMC-NIDDK; L. Radesca, Special Volunteer, LMC-NIDDK; J. Linders, Fogarty Fellow, LMC-NIDDK; S. Richardson, IRTA, LMC-NIDDK.

## COOPERATING UNITS (if any)

Brown University, (W. Bowen); CC-NM (R. Finn, D. Kiesewetter); NHLBI-CH (R. Highet); UCLA Sch. Med. (R. Pechnick); N. S. Kline Inst. Psy. (M. Reith); U of Michigan (J. Woods, C. P. France); MN-NINDS (M. A. Rogawski); BPB-NIMH (A. Pert); M. Herkenham (NIMH-BPB).

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.9

## PROFESSIONAL:

4.9

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Studies are in progress towards the design and synthesis of new phencyclidine (PCP)-like compounds as neuroprotective agents and as anticonvulsants. The design, synthesis, and evaluation of ligands which interact specifically with particular CNS receptors are essential for the elucidation of the function and mechanism of action of these receptors. PCP-like compounds have been reported to exert a protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. We have found that PCP binding sites exist in excitatory amino acid ion channels regulated by glutamate receptors of the N-methyl-D-aspartate (NMDA) type, as well as in the dopamine uptake complex. Our demonstration of the existence of a high affinity PCP binding site associated with the dopamine reuptake carrier raises the possibility that the therapeutic and psychotomimetic effects of PCP in humans are separable and mediated via different binding sites. Metaphit, our electrophilic affinity ligand for PCP binding sites appears to form covalent bonds with macromolecules in these binding sites.

Sigma receptors, are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs and have been implicated in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. We have developed new, potent and selective ligands for the sigma receptor. (+)-Pentazocine has been found to have 5,600-fold selectivity for sigma receptors over PCP receptors. Our synthesis of [3H](+)-pentazocine should greatly facilitate studies of sigma receptor sites. Further, we have synthesized 1S,2R(-)-cis-N-methyl-N-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine. It has been found to be the most potent and selective known sigma receptor ligand, and represents the first member of a novel class of highly potent and selective sigma receptor probes which will be important for further evaluation of the structure and function of this receptor.

Cannabinoid receptors were, also, studied and we have noted that the receptor characterized in our in vitro assay is the same receptor that mediates behavioral and pharmacological effects of cannabinoids, including human subjective experience.

## ANNUAL REPORT OF THE

### PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

#### INTRODUCTION

The Branch activities primarily concern the investigation of the origin, development and natural history of non-insulin-dependent diabetes and its complications in the American Indian population of the United States, and in particular among the Pima Indians of Arizona. Epidemiologic, clinical, and laboratory investigations are conducted by the Branch, which is based in Phoenix, Arizona. The Branch facilities include a field clinic located in the Indian Health Service Hospital at Sacaton, Arizona on the Gila River Indian Reservation and a clinical research center in the Indian Medical Center in Phoenix, Arizona. The Branch consists of two main sections, the Clinical Diabetes and Nutrition Section, which conducts clinical research and laboratory investigations and the Diabetes and Arthritis Epidemiology Section, which is involved primarily in the epidemiological and genetic studies based on the Pima Indian population of the Gila River Indian Reservation. The Branch also serves as a WHO Collaborating Centre in Diabetes, the mission of which is to promote and assist other units and collaborating centres in the methodology and design and analysis of epidemiological and clinical research in non-insulin-dependent diabetes mellitus.

The Clinical Diabetes and Nutrition Section continues to focus on understanding of the role and etiology of non-insulin-dependent diabetes and the investigation of the determinants of obesity. A major longitudinal study of the metabolic characteristics that predict the development of diabetes and of the sequence of events that occur before and during the development of non-insulin-dependent diabetes has continued. This study has demonstrated that reduced insulin mediated glucose disposal or insulin resistance is recognizable and precedes the development of both impaired glucose tolerance and diabetes itself. Insulin resistance has previously been demonstrated by the Section to show familial aggregation and during the past year evidence that insulin resistance may be the result of expression of a major co-dominant gene has been presented. Previous evidence that insulin resistance is a manifestation of an abnormality in the glycogen synthesis pathway together with evidence that the accompanying hyperinsulinemia represents a compensatory response to insulin resistance has led to further studies of the cellular events that characterize insulin resistance in skeletal muscle, which is the primary site of glucose disposal under conditions of hyperinsulinemia where insulin resistance is most clearly demonstrable. Biochemical studies have demonstrated that the site of abnormality is beyond the insulin receptor and indeed collaborative studies with the Diabetes Branch have demonstrated that the Pima insulin receptor in insulin resistant subjects has a normal structure. Similarly tyrosine kinase activity in insulin resistant subjects appears to be quite normal. Studies which are detailed in the Section report describe recent efforts to pinpoint the site of defects in glycogen synthase activation.

The Section has also continued investigations of the determinants of obesity. These have shown that a low metabolic rate and a low ratio of fat to carbohydrate oxidation predict subsequent gain in body weight. Recent data suggest that decreased sympathetic nervous system mediated energy expenditure may contribute to the low metabolic rate and predisposed to obesity. The measurement of energy expenditure in the free-living state can now be investigated as the Section has adapted and refined the method using doubly-labeled water to measure energy

expenditure in man. This method combined with measurements of resting metabolic rate provide a new method to determine the energy costs of physical activity in the free-living state and thus potentially represent a major step in understanding of the determinants of total energy expenditure in an objective and quantitative manner.

The Diabetes and Arthritis Epidemiology Section has continued longitudinal studies of risk factors for diabetes and its vascular complications. During the past year the Section has collected and transformed lymphocytes from members of informative families that are now being used to search for major genes related to the susceptibility to develop diabetes using restriction fragment length polymorphism probes of known chromosomal location and linkage analysis. Investigations of insulin levels have shown fasting hyperinsulinemia is present in Pima Indian children as early as five to six years of age, thus establishing that hyperinsulinemia is present many years before abnormalities of glucose tolerance are detectable. Furthermore studies have shown that the increased immunoreactive insulin levels in those predisposed to develop diabetes but with normal and impaired glucose tolerance are not attributable to an increase in the ratio of proinsulin to true insulin, thus confirming that true hyperinsulinemia in those with normal glucose tolerance is predictive of the subsequent development of non-insulin-dependent diabetes.

A major study of diabetic nephropathy has been established in which groups individuals with normal and impaired glucose tolerance and recent onset and longer duration of diabetes with and without evidence of excessive albumin excretion will be followed with serial measurements of various parameters of glomerular function. These studies will establish the sequence of events that occurs during the development of diabetic nephropathy. Based on cross-sectional analyses it appears that in non-insulin-dependent diabetes these may differ somewhat from those described in insulin-dependent diabetes. Evidence that diabetic nephropathy and end-stage renal disease may have specific genetic determinants that are independent of those of diabetes has been presented. Further studies of the determinants of coronary heart disease and periodontal disease have been completed during the past year. In addition the Section has continued work on the epidemiology of cholelithiasis and rheumatoid arthritis.

Collaborative studies with a number of other diabetes centers and WHO Collaborating Centres in Diabetes has continued. The Branch has completed analyses of serum and urine collected worldwide for the WHO Multinational Study of Diabetes and Its Complications and a further mortality study of the Pima Indian cohort who are one of the component populations in this study has been performed. Members of the Branch have continued to be active in both national and international activities as detailed in the individual section summaries.

#### Invited Lectures and Invited Participation in Symposia

Peter H. Bennett, M.B., F.R.C.P., F.F.C.M.

"Diabetes in American Indians an Overview," Symposium on Diabetes in American Indians and Alaska Natives sponsored by the NIDDK and Indian Health Service, Mesa, Arizona, November 14-17, 1989.

"Etiology of Diabetic Renal Disease in the Pima Indian," Joslin Clinic, Boston, Massachusetts, April 12, 1990.

"The Etiology and Natural History of NIDDM in the Pima Indians," Berson Memorial Lecture, National Institutes of Health, Bethesda, Maryland, May 18, 1990.

"The Prevention of Non-Insulin-Dependent Diabetes," Council on Epidemiology and Health Statistics, American Diabetes Association 50th Annual Meeting, Atlanta, Georgia, June 16, 1990.

"Etiology and Natural History of Glucose Intolerance," Plenary Symposium on Epidemiology of Diabetes, American Diabetes Association 50th Annual Meeting, Atlanta, Georgia, June 17, 1990.

#### Editorial Activities

Associate Editor, American Journal of Epidemiology  
Editorial Board, Diabetes Care  
Associate Editor, Journal of Public Health Medicine

#### Foreign Activities

##### Invited Lectures:

"The Epidemiology and Pathogenesis of NIDDM in the Pima Indians," Swedish Diabetes Association 2nd Annual Meeting, Stockholm, Sweden, October 12-13, 1989.

"Epidemiology of Diabetic Nephropathy and IDDM and NIDDM," Ares Sero Symposium, Lisbon, Portugal, April 16-19, 1990.

"Risk Factors and Determinants of Non-Insulin-Dependent Diabetes," China-Japan Friendship Hospital, Beijing, China, April 24, 1990.

"Diabetic Nephropathy," DaQing First Hospital, DaQing, China, April 27, 1990.

"What We Have Learnt From Studies of Non-Insulin-Dependent Diabetes Among the Pima Indians of Arizona," Journees de Diabetologie De l'Hotel-Dieu, Paris, France, May 11, 1990.

Resident Faculty, World Health Organization/International Diabetes Federation Course on Epidemiological and Public Health Aspects of Diabetes Mellitus, Cambridge, England, July 15-28, 1990.

#### Other Activities:

Co-organizer of NIDDK and Indian Health Service Symposium on Diabetes in American Indians and Alaska Natives, Mesa, Arizona, November 14-17, 1989.

Member, National Diabetes Data Group

Member, Scientific Program Committee, 14th International Diabetes Federation  
Chairman, Organizing Committee, Epidemiology Satellite Meeting of 14th International Diabetes Federation Congress

## Clinical Diabetes and Nutrition Section

The scientific mission of the CDNS, NIDDK, is to determine the etiology and pathogenesis of non-insulin dependent diabetes mellitus (NIDDM) as it occurs among the Pima Indians of Arizona. Our research efforts, therefore, have been in three broad categories. Firstly, we are conducting a cross-sectional and longitudinal study of the development of NIDDM among non-diabetic Pima Indians with the aim to determine the earliest detectable abnormality associated with the development of the disease and to also carefully define the natural history of the evolution of the various metabolic defects of the disease as the hyperglycemia evolves. Secondly, since insulin resistance appears to be a major determinant of the development of NIDDM, we are conducting biochemical and molecular studies to determine the specific abnormality that causes insulin resistance among the Pimas. Thirdly, since obesity is such a major risk factor for the development of NIDDM, we are conducting studies to understand the etiology of obesity.

Cross-sectional and longitudinal study of the development of NIDDM. Beginning in 1982, a longitudinal study of approximately 300 Pima Indians was undertaken to 1) determine the metabolic characteristic which is most predictive of subsequent development of NIDDM among non-diabetics and 2) document the sequence of metabolic events that occurs during the transition from normal to impaired glucose tolerance and subsequently to marked fasting hyperglycemia. Nearly 300 individuals were entered into this study with each of these individuals to be restudied on a yearly basis. Considerable information has been obtained from the cross-sectional analyses of these data and now, with over 30 individuals having developed diabetes during the course of our observations, we are now in a position to analyze formally these data using survival analysis techniques to define the risk factors that are most predictive of the development of NIDDM in this population.

In addition, as part of these studies, we have studied a group of Caucasians to make comparisons between the two racial groups. It is apparent from these studies that the Pima Indians are more insulin resistant at a given degree of obesity than Caucasians and that at similar degrees of obesity and insulin resistance, the Indians have greater insulin secretion. The increased insulin secretion was particularly evident at early time points following oral or intravenous glucose. This suggests that at least one of the major abnormalities predisposing the Pimas to NIDDM as compared to Caucasians is their greater degree of insulin resistance.

Our preliminary analyses among the Pimas have also suggested that insulin resistance will be a major risk factor for the development of NIDDM. The insulin resistance, however, is not determined completely by the degree of obesity and there is strong familial aggregation of insulin resistance independent of degree of obesity, age, and sex. The familial aggregation of the disease is consistent with genetic determinants, although environmental factors certainly play a role and cannot be excluded on the basis of familiarity. However, also consistent with the genetic hypothesis is that insulin resistance, particularly at maximally-stimulating insulin concentrations in vivo, appears to be distributed as a mixture of three normal distributions rather than a single normal distribution. This is suggestive of a single, major autosomal gene, inherited in a codominant manner, that is a significant determinant of insulin resistance in the population. Preliminary analyses of the natural history of the disease



have demonstrated that the transition from normal glucose tolerance to impaired glucose tolerance is associated with a worsening of insulin resistance and heightened insulin secretion. The insulin secretion appears to be in no way deficient in this transition and is as expected for the degree of insulin resistance that develops. In marked contrast, however, the transition from impaired glucose tolerance to NIDDM is associated primarily with a deterioration of insulin secretion. These preliminary observations will have to be confirmed over the next several years as larger numbers of individuals are studied at several different points during the evolution from normal glucose tolerance to NIDDM.

As a corollary to this work, we have pursued investigations as to the mechanism of the insulin resistance associated with body weight gain and obesity. Previous work in our laboratory had demonstrated a significant negative linear correlation between degree of insulin resistance in the physiologic range of insulins and skeletal muscle capillary density. It is therefore postulated that since the unfenestrated capillaries of muscle are relatively impermeable to insulin, the increased insulin resistance in those with low capillary density might be due to altered kinetics of insulin penetration to its sites of action in muscle. To test this hypothesis, a method was developed for directly collecting lymph from peripheral lymphatics that primarily drain skeletal muscle tissue. Preliminary results of these data suggest that during insulin infusion in vivo insulin concentrations in lymph rise much more slowly than arterial concentrations, and even after 2-1/2 hours are still about 30% of the levels obtained in the artery. These data suggest that the capillary endothelium may provide a true physiologic block to the transport of insulin from the artery to the target tissues and therefore constitute a potential site for regulation of insulin action. Whether this block is altered in various stages such as obesity or diabetes remains to be determined.

Insulin resistance. Since insulin resistance appears to be a risk factor for the development of NIDDM in the population, in vitro studies have been undertaken to determine the site of this defect in the transduction of the insulin signal. Previous work in our laboratory has established that there is a very good correlation between insulin action in vivo and insulin stimulation of skeletal muscle glycogen synthase activity in biopsies obtained from the vastus lateralis muscle during insulin infusion in man. Our efforts have therefore now focused on determining the reason for the dysregulation of glycogen synthase activity in insulin-resistant individuals. This effort has included studies of skeletal muscle insulin receptor binding and tyrosine kinase activity as well as other enzymes known to be involved in the glycogen synthetic pathway and enzymes in other metabolic pathways that are insulin-regulated in skeletal muscle in man.

It was found that the insulin resistance in Pima Indians with impaired glucose tolerance could not be explained by an abnormality of insulin binding in skeletal muscle and that the insulin regulation of the tyrosine kinase activity of the beta-subunit of the receptor was also normal in these insulin-resistant subjects. In fact, if anything, there appeared to be an up-regulation of the tyrosine kinase activity of the receptor in these insulin-resistant individuals, as if there was some compensatory response to a metabolic defect beyond the receptor. Consistent with this observation is the fact that the insulin regulation of casein kinase II activity in skeletal muscle from insulin-resistant subjects was also greater than that observed among insulin-sensitive subjects. Thus the putative metabolic defect that causes insulin resistance among the Pimas appears

not to be at the level of the insulin receptor and not in the pathway between the receptor and its activation of casein kinase II activity.

On the other hand, abnormalities have been observed in several other metabolic enzymes in skeletal muscle of insulin-resistant subjects. Firstly, it was found that the fasting glycogen synthase phosphatase activity was reduced in insulin-resistant subjects. In addition, insulin stimulation of the enzyme occurred in insulin-sensitive subjects and peaked at approximately 10 minutes following insulin infusion, but that insulin stimulation failed to occur in the insulin-resistant individuals. Similar results were obtained using phosphorylase rather than exogenous rabbit glycogen synthase as substrate. Using specific inhibitors, the abnormal enzyme activity was identified as a type 1 phosphatase and the reduced activity could be observed in both glycogen and cytosolic subcellular fractions, suggesting an alteration in the catalytic subunit independent of the glycogen-bound regulatory subunit or G-component. This abnormality in fasting type 1 phosphatase activity in insulin-resistant individuals persisted following trypsin treatment, suggesting that the decreased activity was not a result of the heat-stable inhibitors 1 and 2. Immunoblots indicated that the reduced fasting activity was not due to a decrease in catalytic subunit concentration, suggesting that the intrinsic activity of the catalytic subunit or regulation of its activity is abnormal in insulin-resistant subjects.

Studies have been carried out on the insulin regulation of cyclic AMP-dependent protein kinase in the skeletal muscle of insulin-resistant subjects. These studies demonstrated that in insulin-sensitive subjects there was a reduced apparent affinity of the regulatory subunit of this enzyme for cyclic AMP following an insulin infusion and that decrease in apparent sensitivity did not occur in insulin-resistant subjects. These results suggest that, in addition to the abnormal glycogen synthase phosphatase abnormality, there was an abnormality in insulin regulation of cyclic AMP-dependent protein kinase activity that may contribute to the abnormal regulation of glycogen synthase activity.

In addition to these studies of enzymes known to be involved in the glycogen synthetic pathway, studies were undertaken to look at other pathways that were known to be regulated by insulin in other systems. Specifically, we investigated the effect of insulin on S6kinase activity in skeletal muscle from insulin-resistant and insulin-sensitive Pima Indians. The basal S6kinase activity was similar in the insulin-resistant and -sensitive groups, but although insulin stimulated S6kinase activity increased sharply between 15 and 30 minutes of insulin infusion in the insulin-sensitive individuals, there was quite a different pattern observed in insulin-resistant subjects. In these subjects, the maximum stimulation was delayed and occurred at 60 minutes. This absence of the early insulin-stimulated response in the insulin-resistant subjects suggested that there may be an absence of a particular S6kinase activity while a later activating kinase was responding normally. Chromatography on FPLC MonoQ revealed two peaks of insulin-stimulated S6 activity that eluted identically in both the insulin-sensitive and insulin-resistant groups. Immunoblot analysis showed that the enzyme responsible for the first peak of activity was antigenically related to a 90 kilodalton S6kinase and the activity in the second eluted peak was antigenically related to a 70 kilodalton S6kinase. The abnormal timecourse of S6 activation in the insulin-resistant subjects did not appear to be explained by a lack of one of these two kinases but rather by a lower magnitude and rate of stimulation of these activities.

Studies have also been carried out of insulin regulation of protein tyrosine phosphatase activity in muscle extracts from insulin-sensitive and insulin-resistant individuals. The pre-insulin or basal PTPase activity in the soluble fraction of the extract was the same in sensitive and resistant subjects, but in the particulate fraction, the basal activity was 30% higher in resistant subjects. Soluble PTPase activity was rapidly suppressed by about 25-30% following insulin infusion in insulin-sensitive subjects. Conversely, the effect of insulin appears to be greatly reduced in insulin-resistant individuals.

Finally, we have recently begun studies of insulin's effect on gene transcription. Specifically, we are studying the ability of insulin to regulate transcription of the insulin-sensitive glucose transporter gene and of the proto-oncogene, c-fos. The project will involve identification of insulin-sensitive cis-acting elements in the promoter region of these genes and then subsequent characterization of putative transacting factors that may bind to these elements and mediate the effects of insulin on transcription. Comparisons will be made between these factors and elements between insulin-sensitive and insulin-resistant Pima Indians.

Obesity. Obesity is extremely prevalent among the Pima Indians and is a major risk factor for the development of NIDDM. Our studies in this area have been to identify etiologic factors leading to the development of obesity, thereby identifying factors which are important risk factors for the subsequent development of NIDDM.

We have previously observed, using indirect calorimetric methods both in a respiratory chamber and using a ventilated hood, that the metabolic rate varies between individuals more than can be explained by individual differences in body size, body composition, age, and sex. Metabolic rate aggregates in families independent of these covariates as well as does the rate of fuel mix oxidation, suggesting that there may be genetic components to both the whole-body metabolic rate as well as the rate of oxidation of carbohydrate versus lipid. Several factors have been excluded as being major determinants of energy expenditure. Specifically, age does not appear to be a major determinant other than through its effect on the lean body mass; the degree of physical fitness does not change energy expenditure beyond the differences that occurred in its effect on body weight and body composition.

Most importantly in longitudinal studies, a low metabolic rate as well as a low ratio of fat to carbohydrate oxidation were demonstrated to be specific risk factors for the subsequent gain in body weight. Because of these observations, efforts were undertaken to pursue the causes of differences in metabolic rate between individuals.

The sympathetic nervous system activity, as assessed by measuring urinary catecholamines, as well as the effect of propranolol, a beta-blocker, has suggested that part of the variability in the metabolic rate among Caucasians is dependent on beta-adrenergic stimulation, whereas this was not found to be true among the Pima. These data suggest that decreased sympathetic nervous system-mediated energy expenditure may contribute to abnormalities in energy expenditure among the Pimas and possibly predispose them to obesity.

Most studies of metabolic rate and weight gain, however, were conducted in the fairly restricted conditions of the metabolic ward. To study energy expenditure

in the more free-living conditions, we have adapted the method using doubly-labeled water to measure energy expenditure among the free-living Pima Indian population. This was first accomplished by a revalidation of the method by comparing the results of the metabolic rate measured on individuals in our respiratory chamber to those of the doubly-labeled water method. These two methods were found to be quite closely correlated, such that the doubly-labeled water method should provide an acceptable means of measuring physical activity in the free-living condition. Furthermore, by studying individuals both in the respiratory chamber and free-living conditions, the difference between these two energy expenditure measurements should provide us with a measure of the energy cost of physical activity in an individual. This will then provide the first opportunity to look directly at the variability of the energy cost of physical activity in relatively sedentary populations, how this may differ between individuals, and whether it has any specific predictive value for subsequent weight gain.

Finally, to explore the mechanisms for the variation in the rate of fuel mix oxidation between individuals, we have begun studies of insulin regulation of lipoprotein lipase activity in skeletal muscle and adipose tissue from individuals with a high ratio of carbohydrate to fat oxidation rates versus those with low rates of carbohydrate to fat oxidation rates. These studies should provide information about the mechanisms of control of fuel mix oxidation rates.

#### Awards

Clifton Bogardus, M.D.

Outstanding Service Medal, PHS Commissioned Corps, 1989

Public Health Service Superior Service Award, 1990

#### Invited Lectures and Invited Participation in Symposia

Clifton Bogardus, M.D.

"Presentation of the ADA Position Paper on Diabetes and Exercise," American Diabetes Association 50th Annual Meeting and Scientific Sessions Exercise Symposium, Atlanta, Georgia, June 16, 1990.

"In pursuit of the gene(s) of NIDDM," and "Metabolic basis of obesity," American Diabetes Association 50th Annual Meeting and Scientific Sessions symposium Atlanta, Georgia, June 18, 1990.

"Predictions from the phenotype and natural history of NIDDM," 71st Annual Session of the American College of Physicians, Chicago, Illinois, April 4, 1990.

"Pathophysiology of NIDDM in Pima Indians," University of California, San Francisco Endocrine Grand Rounds, San Francisco, California, April 4, 1990.

"Studies of the determinants of insulin resistance in Pima Indians," San Francisco Veterans Administration Hospital Endocrine Metabolism Conference, San Francisco, California, April 4, 1990.

"Metabolic determinants of obesity in man," Medical Grand Rounds, University of Alabama, Birmingham, Birmingham, Alabama, March 22, 1990.

"Pathogenesis of non-insulin dependent diabetes mellitus," University of Alabama, Birmingham Endocrine Conference, Birmingham, Alabama, March 22, 1990.

"Pathogenesis of non-insulin dependent diabetes mellitus among the Pima Indians," Indiana University Medical Center Endocrine Research seminar series, Indianapolis, Indiana, February 26, 1990.

"Insulin resistance and the pathogenesis of non-insulin dependent diabetes mellitus," Endocrine Grand Rounds, Massachusetts General Hospital, Boston, Massachusetts, December 19, 1989.

American Diabetes Association Postgraduate Workshop, Orlando, Florida, January 11, 1990. Dr. Bogardus participated in a debate with Dr. Dean Lockwood on the topic of insulin therapy in the obese patient with diabetes.

Stephen Lillioja, M.B., Ch.B., F.R.A.C.P.

"Diabetes in the Pima Indians," Amylin Corporation, San Diego, California, February 17, 1990.

Eric Ravussin, Ph.D.

"State-of-the-art methods to measure energy expenditure in man," Smoking and Body Weight Workshop, Memphis, Tennessee, September 11, 1990.

"Energy expenditure measurements in children," Children's Nutrition Research Center, Houston, Texas, July 23, 1990.

"Energy expenditure in the pathogenesis of obesity," Symposium on Obesity, Energy Expenditure, and Body Weight Standards at the Annual Meeting of the Federation of American Societies for Experimental Biology, Washington, D.C., April 2, 1990.

"The genesis of obesity in the adult," Third Annual Conference on Clinical Nutrition, San Jose, California, March 7, 1990.

"Energy efficiency and relationship to body weight changes," American Society for Parenteral and Enteral Nutrition Clinical Congress, San Antonio, Texas, January 28, 1990.

"Energy cost of spontaneous physical activity," Annual Meeting of the Southwest Chapter of the American College of Sports Medicine, San Diego, California, December 2, 1989.

James Sommercorn, Ph.D.

"Regulation of Casein Kinase II by Insulin," Cellular and Molecular Biology Program seminar series, Arizona State University, Tempe, Arizona, December 4, 1989.

## Foreign Activities

Eric Ravussin, Ph.D.

Invited speaker at:

"Depense energetiques: facteur predictif de la prise de poids," Journees de Diabetologie de l'Hotel-Dieu de Paris, Paris, France, May 10, 1990.

"Reduced rate of energy expenditure as a risk factor for body weight gain," III International Congress on Obesity, Rio de Janeiro, Brazil, April 28-29, 1990. Dr. Ravussin also debated with Dr. George Bray on the topic "Are the obese guilty?"

"The measurement of energy expenditure and its clinical implications," Katholieke Universiteit Leuven, LEGENDO, Leuven, Belgium, December 11, 1989.

"Metabolisme energetique et obesite," Forum Lavoisier, Paris, France, December 8, 1989.

"Genetics and obesity," Scientific Conference of the World Sugar Research Organization, Sao Paulo, Brazil, October 11, 1989.

## Diabetes and Arthritis Epidemiology Section

The Diabetes and Arthritis Epidemiology Section has continued the longitudinal studies of genetic and environmental risk factors for diabetes and its vascular complications in the Pima Indians, as well as continuing epidemiologic studies of rheumatoid and other forms of arthritis, cholelithiasis, mortality rates and causes of death.

### Natural history and risk factors for non-insulin-dependent diabetes.

The long follow-up provided by the Pima Indian study yields increasingly valuable data on complications of diabetes and the transmission of susceptibility to diabetes and its complications from one generation to the next. At least part of the susceptibility to diabetes appears to be transmitted by a major autosomal gene, the location of which is being sought by means of linkage analysis. To this end DNA samples have been collected and EBV-transformed lymphocyte cultures have been established from members of families informative for linkage analysis.

Fasting hyperinsulinemia and obesity in Pima children are early abnormalities which ultimately lead to diabetes and which are strongly familial, and probably genetic. Although these factors in children are associated with maternal diabetes in pregnancy, they do not fully account for the greatly increased risk of diabetes in the offspring of diabetic pregnancies. Thus the diabetic intrauterine environment has additional deleterious effects ultimately leading to diabetes.

Previous studies of the increase and subsequent decline in serum insulin concentrations during the transition from normal to impaired glucose tolerance (IGT) to diabetes have been extended. The increased insulin concentration in IGT is not due to an increased proinsulin-insulin ratio and is not dependent on weight gain. Thus it probably reflects increasing insulin resistance which is

not entirely attributable to obesity. Lower post-load insulin concentrations in IGT predict worsening to diabetes. Thus insulin resistance, partially compensated by hyperinsulinemia, leads to IGT, and insulin secretory failure results in worsening to diabetes. Whether these two metabolic defects result from one or two genetic defects (probably interacting with environmental factors) remains unknown. The finding of islet amyloid polypeptide in most autopsied diabetic subjects is compatible with a primary islet cell lesion, but could also be secondary to diabetes.

Complications of Diabetes. Diabetes complications are being documented and their risk factors determined. Major complications under study are nephropathy, end stage renal disease, retinopathy, coronary heart disease (CHD), lower extremity amputation, and periodontal disease, all of which are related to the duration of diabetes. All of these complications except CHD develop at least as frequently in this population with non-insulin-dependent diabetes as in people with insulin-dependent diabetes. Among Pimas, the frequency of nonfatal CHD is greatly increased by diabetes, and fatal CHD occurs almost exclusively in those with diabetes. As shown previously for other complications of diabetes, one of the strongest risk factors for CHD is insulin treatment, suggesting either that exogenous insulin increases susceptibility to a wide variety of vascular complications of diabetes, or that patients who receive insulin treatment are at higher risk of vascular disease because of inherently more severe hyperglycemia or other unknown factors.

Overt diabetic nephropathy, defined by levels of proteinuria previously shown to be strongly predictive of renal failure or death, can now be predicted by mildly elevated urinary albumin excretion ("microalbuminuria"), which is often present early in diabetes and in some cases even precedes diabetes. The possibility that susceptibility to diabetic nephropathy may be identified early, and possibly even before the onset of diabetes, has important implications for genetic and preventive studies.

In collaboration with investigators from Stanford University and The Cleveland Clinic, we have begun a detailed study of renal function in Pimas with normal or impaired glucose tolerance or diabetes of short or long duration. These groups will be compared with respect to renal plasma flow, glomerular filtration and glomerular pore size distribution, and these functional measures will be related to blood pressure, degree of hyperglycemia, and disease progression during subsequent follow-up.

Diabetic nephropathy among Pimas with diabetes is highly familial, whether nephropathy is defined as heavy proteinuria or as renal insufficiency. The genetic approaches described above for diabetes will also be applied to studying the genetics of diabetic nephropathy.

Other activities. Section staff continue to be active in medical research and activities beyond the projects described here. Staff collaborate extensively in research projects conducted by the Clinical Diabetes and Nutrition Section and the National Center for Health Statistics. They contribute to national and international meetings and workshops. The Section Staff participated in the following national and international activities:

### Invited Lectures and Invited Participation in Symposia

William C. Knowler, M.D., Dr.P.H.

"Determinants of NIDDM," Symposium on Diabetes in American Indians and Alaska Natives, sponsored by the NIDDK and Indian Health Service, Mesa, Arizona, November 14-17, 1989.

"Diabetes mellitus in the Pima Indians," Division of Endocrinology, University of Iowa, Iowa City, Iowa, June 1, 1990.

Invited Speaker and Session Chairman, Workshop on Obesity and Cardiovascular Disease in Minority Populations, NIH, August 28-29, 1990.

David J. Pettitt, M.D.

"Pregnancy and Diabetes," Symposium on Diabetes in American Indians and Alaska Natives, sponsored by NIDDK and Indian Health Service, Mesa, Arizona, November 14-17, 1989.

### Foreign Activities

William C. Knowler, M.D., Dr.P.H.

Visiting Professor, Department of Community Health Sciences, Lund University, Dalby, Sweden, July 1990 - (Funded by the Medical Research Council of Sweden).

"High blood pressure and the complications of diabetes: which comes first?" Symposium on Hypertension and Diabetes, sponsored by the International Society of Hypertension and the Chinese University of Hong Kong, Hong Kong, February 9-10, 1990.

David J. Pettitt, M.D.

"Long-term effects of the diabetic pregnancy on the offspring," Upsalla University, Upsalla, Sweden, September 18, 1990.

### Biostatistics and Data Management Section

This section is engaged in data management and support activities for the research operations of the Branch as a whole. The major activities are updating, error checking, storage, and retrieval of datasets for the extensive epidemiologic study; providing a computer-based work scheduling system for the radio-immunoassay laboratory; and assistance with many datasets from the studies conducted by the Clinical Diabetes and Nutrition Section. Other major activities include support of laboratory instrument-computer interfacing, adaptation of genetics programs written by non-NIH scientists, and extensive support of microcomputers, including installation of local area networks in Phoenix and Sacaton.

The use of microcomputers is being expanded so that many tasks are now done more easily, at lower cost, or with greater accuracy than was previously possible. Applications include the use of computer graphics for most scientific



presentations and publications and a microcomputer-based system in the outpatient research clinic in Sacaton, Arizona for maintenance and updating of demographic data for the entire study population and the scheduling of research procedures in the clinic.

Consulting on statistical and epidemiologic methods and data management for specific scientific projects has been the other major activity of the Section, on which much of the productivity of the direct research activities of the Branch depends.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Diabetes Mellitus and Other Chronic Diseases in the Gila River Indian Community

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	D. Mott	Research Chemist	CDNS, NIDDK
	W.J. Butler	Computer Systems Analyst	BDMS, NIDDK

## COOPERATING UNITS (If any)

Biostat. and Data Management Sec., Clinical Diabetes and Nutrition Sec., PECCR, NIDDK; Indian Health Service; Ariz. State U.; Cleveland Clinic, Cleveland OH; University of Pittsburgh.

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

1.9

## PROFESSIONAL:

1.1

## OTHER:

0.8

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to identify the determinants of non-insulin-dependent diabetes (NIDDM), various types of arthritis, and gallbladder disease, and elucidate the natural history of the diseases. Genetic and environmental risk factors for NIDDM have been studied in the Pima Indians. The residents of the study area, approximately 5000 people, have participated in a longitudinal population study for the last 25 years, allowing observations of the natural history of diabetes mellitus. Risk factors for obesity, hypertension, and cholelithiasis are also studied, along with the relationships of these diseases to diabetes and their effects on mortality rates. The genetics of diabetes is studied by means of family studies and relationships of genetic markers to disease. The roles of obesity, serum insulin concentrations, impaired glucose tolerance, occupational and leisure-time physical activity and diabetes in relatives are assessed. The severity of abnormality of glucose homeostasis is assessed by measurement of plasma glucose and serum insulin concentrations during glucose tolerance tests and measurement of glycosylated hemoglobin. This study has shown diabetes to be a serious and common disease with both genetic and environmental components. Hyperinsulinemia, reflecting insulin resistance, is an early abnormality predicting diabetes. The deterioration from impaired glucose tolerance to diabetes may be precipitated by insulin secretory failure.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D.J. Pettitt Assistant Chief DAES, NIDDK

Others:	P.H. Bennett	Chief	PECRB, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Biostatistics and Data Management Section, PECRB;  
Karolinska Institut, Stockholm, Sweden (B. Perrson) (Foreign);  
Mayo Clinic, Rochester, Minnesota (B.A. Kottke)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.7

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Macrosumia, prematurity, perinatal mortality, and congenital malformations are more common in infants of diabetic mothers than in infants of nondiabetic mothers. Offspring of diabetic women are also at an increased risk of developing obesity and glucose intolerance during childhood and young adulthood. The purposes of the project are to identify diabetes and impaired glucose tolerance during pregnancy in women in the Gila River Indian Community, to determine the effects of abnormal glucose tolerance on outcome of the pregnancy, and to determine long term prognosis for the women and their offspring. By means of a glucose tolerance test as well as chart review, the diabetes status of every woman is determined at two-yearly intervals and during the third trimester of each pregnancy. The characteristics of women who have diabetes or impaired glucose tolerance during the pregnancy are compared to those of women who are normal during the pregnancy and subsequently develop diabetes and to those of women who remain normal. These women and their offspring, after the age of 5 years, are followed at two yearly intervals and glucose tolerance tests are performed which include measurements of glucose and insulin. Offspring of diabetic women have more diabetes and more obesity than offspring of nondiabetic and prediabetic women. Fasting serum insulin concentrations were compared in nondiabetic offspring of nondiabetic, prediabetic, and diabetic women. Even after adjustment for fasting plasma glucose, the offspring of prediabetic and diabetic women had significantly higher insulin concentrations than offspring of nondiabetic women. In spite of the previously described effect of the intrauterine environment on the risk for obesity and diabetes, these data suggest that insulin resistance, as estimated by the fasting insulin concentration, is an inherited trait and is not induced simply by the exposure to the diabetic intrauterine environment.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Muscle Capillary Basement Membrane Thickness Prior to Onset of Diabetes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK  
W.C. Knowler Chief DAES, NIDDK

## COOPERATING UNITS (if any)

Department of Biology, Case Western Reserve University, Cleveland, Ohio (N.B. Rushforth) and Department of Medicine, University of California, San Francisco, California (M.D. Siperstein)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.0

## PROFESSIONAL:

0.0

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gila River Indian Community Autopsy and Mortality Study

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.L. Sievers	Guest Researcher	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK

## COOPERATING UNITS (if any)

Pathology Department, Phoenix Indian Medical Center, Indian Health Service, Phoenix, Arizona; Cleveland Clinic Foundation, Phoenix, Arizona; Radcliffe Infirmary, Oxford, UK (Foreign).

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.4

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☒ (b) Human tissues
 ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The causes of death and postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects. Medical records are reviewed for the determination of cause of death and for the occurrence of certain serious diseases or complications of diabetes.

The purpose of the study is to relate the outcome and cause of death to events or risk factors measured in life among Pima Indian residents of the Gila River Indian Community, particularly in relation to diabetes, cardiovascular diseases and gallbladder disease. Post-mortem examinations are obtained whenever possible on members of the Gila River Indian Community to ascertain conditions present at the time of death and ascertain cause of death as precisely as possible. In addition, death certificates and all available medical records pertaining to the subjects are obtained and reviewed in a standardized way for evidence of the complications of diabetes, vascular disease, neoplasms and other conditions, which may have been recognized prior to death. The records of the occurrence of such conditions, together with conditions recognized at autopsy, are used to determine the causes of death and incidence of complications associated with diabetes and other conditions identified initially during life by the longitudinal epidemiologic studies in the population.

The pancreatic sections from autopsied Pimas were examined for peptide hormones and amyloid. Islet amyloid was found in 77% of the diabetic and 8% of nondiabetic subjects, suggesting that islet amyloid may reflect a primary beta cell defect contributing to diabetes, or may develop as a result of secondary beta cell dysfunction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69009-25 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Natural History of Arthritis and Rheumatism in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: D.J. Pettitt Assistant Chief DAES, NIDDK  
W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Biostatistics and Data Management Section, PECRB, Arizona State University;  
University of Alabama at Birmingham.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.4

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development and progression of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis are being determined by means of clinical, radiographic and serological examinations carried out prospectively at two-yearly intervals among adults of the Gila River Indian Community (Pima Indians) in Arizona, in conjunction with epidemiological studies of diabetes in the same community. The purpose of this investigation is to ascertain the determinants of these diseases in the population, and to identify factors which alter the natural history of progression of the disease. Host factors such as age, sex, and various gene markers including HLA and Gm, together with various potential environmental determinants, such as obesity and evidence of exposure to infectious agents, will be studied prospectively to determine their relationship to the development of these diseases. Longitudinal data have now been collected for 25 years and represent a unique data set for epidemiological studies of arthritis.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cross-Sectional and Longitudinal Study of "Prediabetes" in the Pima Indians

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist with tenure	CDNS, NIDDK
	B.L. Nyomba	Visiting Associate	CDNS, NIDDK
	F. Zurlo	Visiting Fellow	CDNS, NIDDK
	M. Saad	Visiting Associate	DAES, NIDDK
	M. DeGregorio	Visiting Fellow	CDNS, NIDDK
	R. Ferraro	Staff Fellow	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes Branch, NIDDK (S. Taylor and J. Roth)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

4.75

## PROFESSIONAL:

3.60

## OTHER:

1.15

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Pima Indians of Arizona have the highest prevalence and incidence rate of non-insulin dependent diabetes mellitus (NIDDM) of any population in the world. We have been studying a subset of this population that is at highest risk of developing the disease for the purposes of 1) to determine the metabolic characteristic(s) which is most predictive of the subsequent development of NIDDM among non-diabetics and 2) to document the sequence of metabolic events that occur at the transition from normal to impaired glucose tolerance and subsequently to marked fasting hyperglycemia and NIDDM. Indian volunteers are admitted to the clinical research ward for about ten days to undergo a variety of tests to assess body composition, oral and intravenous glucose tolerance, insulin secretory dynamics, energy metabolism, and insulin action. To date the results have shown that insulin resistance is a major and significant predictor of the subsequent development of NIDDM among non-diabetic subjects. The transition from normal to impaired glucose tolerance is associated with deterioration of insulin action in vivo and the insulin response to the development of this insulin resistance appears to be appropriate for the degree of glycemia that occurs with the impaired glucose tolerance. Transition from impaired glucose tolerance to marked fasting hyperglycemia is then associated with the deterioration in insulin secretory dynamics. Comparison with Caucasians has shown that Pima Indians are more insulin-resistant at a given degree of obesity. More notable, however, is that even when matched for insulin resistance or obesity, Pima Indians have greater insulin responses to glucose or a meal. The differences are particularly notable at earliest time points after a stimulus and this suggests that poor pancreatic function does not underlay diabetes in this population. In collaboration with other laboratories, the insulin gene and the insulin receptor gene have both been sequenced in Pima Indians and found to be normal.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69020-07 PEGR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance and the Regulation of Muscle Glycogen Synthase Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: B.L. Nyomba Visiting Associate CDNS, NIDDK  
Y. Kida Visiting Fellow CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Division of Biology and Medicine, Brown University, Providence, RI (D. Brautigan)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

0.82

PROFESSIONAL:

0.32

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are currently characterizing the abnormalities for regulation of human muscle glycogen synthase in insulin-resistant subjects. In insulin-resistant subjects, fasting glycogen synthase phosphatase activity is reduced and fails to show the peak insulin stimulation observed for insulin-sensitive subjects at 10 minutes. Similar results were obtained using phosphorylase as substrate for the phosphatase activity with the exception that insulin-sensitive subjects maintained the stimulated level of activity from 20 minutes until the end of the insulin infusion. Using specific inhibitors the abnormal enzyme activity was identified as a type-1 phosphatase in human muscle from insulin-resistant subjects. The reduced type-1 activity observed in both glycogen and cytosolic subcellular fractions suggested an alteration in the catalytic subunit independent of the glycogen-bound regulatory subunit or G component. The abnormally low fasting type-1 phosphatase activity in insulin-resistant subjects persisted following trypsin treatment, suggesting that inhibitors 1 and 2 (characterized regulators of type-1 phosphatase) are not important determinants of the abnormal phosphatase activity. Western blots indicate an increased concentration of catalytic subunit for type-1 phosphatase in the muscle from insulin-resistant subjects. This result suggests that the intrinsic activity or regulation of the catalytic subunit is abnormal in insulin-resistant subjects. The apparent affinity for cAMP activation of muscle cAMP-dependent protein kinase (CPK) is not decreased in insulin-resistant subjects following insulin infusion. In contrast, sensitive subjects show a reduced apparent affinity of their CPK regulatory subunit for cAMP following insulin infusion. This results in inactivation of the kinase and could stimulate glycogen synthase activity. These results suggest that abnormal regulation of both glycogen synthase phosphatase and CPK contribute to the insulin resistance which is secondary to abnormal insulin-stimulated glycogen synthase activity.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69021-10 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Expenditure in Pima Indians: Risk Factors for Body Weight Gain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: E. Ravussin Visiting Scientist CDNS, NIDDK  
R. Ferraro Staff Fellow CDNS, NIDDK  
F. Zurlo Visiting Fellow CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Dept. of Medicine, Harvard Medical School, Boston MA (J. Young and J. Flier)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

1.60

PROFESSIONAL:

1.10

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our investigations of the possible causes of body weight gain in Pima Indians using indirect calorimetry (respiratory chamber and ventilated hood systems) have so far shown that: 1) metabolic rate varies between people more than can be explained by individual differences in body size, body composition, age, and sex; 2) siblings studies have shown family membership to be an important determinant of metabolic rate and fuel mix oxidation, suggesting a genetic component; 3) age is not a major determinant of energy expenditure; 4) physical fitness does not change energy expenditure beyond the differences in body weight and body composition; 5) low metabolic rate as well as a low ratio of fat to carbohydrate oxidation is a risk factor for body weight gain; 6) sympathetic nervous system (SNS) activity is a determinant of metabolic rate in Caucasians but not in Pima Indians, suggesting that decreased SNS-mediated energy expenditure in Pima Indians may predispose them to obesity; 7) large inter-individual differences in energy expenditure are observed in free-living conditions (using the doubly-labeled water technique), suggesting large differences in physical activity; 8) weight gain can be viewed as the means by which the organism compensates for intrinsic metabolic defects. Weight gain is accompanied by an increase in: a) resting metabolic rate beyond the increase in body weight; b) energy cost of physical activity; c) fat mass and therefore fat oxidation; and d) insulin resistance which would promote fat oxidation. All of the above changes would then decrease the rate of weight gain.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69024-04 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) WHO Collaborating Center for Epidemiological and Clinical Investigations in Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: W.C. Knowler Chief DAES, NIDDK  
C. Bogardus Chief CDNS, NIDDK  
D.J. Pettitt Assistant Chief DAES, NIDDK

COOPERATING UNITS (if any) World Health Organization, Non-Communicable Diseases Program, Geneva, Switzerland, (Foreign), Other World Health Organization Collaborating Centers for Diabetes (Foreign), China-Japanese Friendship Hospital, Beijing, China (Pan Xiaoren) (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.3

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Phoenix Epidemiology and Clinical Research Branch was designated as the WHO Collaborating Center for Design, Methodology and Analysis of epidemiological and Clinical Investigations in Diabetes in 1986. The purposes of the Center are to collaborate with the World Health Organization in the implementation of the WHO/IDF action program to provide advice, consultation and collaboration with other investigators in the design, methodology and analysis of epidemiology and clinical investigations relating to the etiology and pathogenesis of non-insulin dependent diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations, and data analysis relating to diabetes and collaborate with those interested in applying such techniques elsewhere. The Center will advise and help in the design of new studies, including onsite assistance when necessary.

The center serves as a central laboratory for the WHO Multicenter Study of Vascular Disease in Diabetes, as well as being a participating center for this study which is examining the mortality and incidence of vascular complications of diabetes among different ethnic groups in different countries. In addition the center has initiated a collaborative study of impaired glucose tolerance in China, and is collaborating in the preparation of a survey manual for diabetes mellitus on behalf of WHO.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Treatment of Impaired Glucose Tolerance in Malmöhus County Sweden

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler

Chief

DAES, NIDDK

## COOPERATING UNITS (if any)

Lund University, Dalby, Sweden (Foreign)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Mortality according to glucose tolerance was studied to determine the prognosis of impaired glucose tolerance. In 1962-65, 228,833 subjects were screened for glycosuria. Of 2477 with glycosuria, 2180 were given oral glucose tolerance tests and grouped according to normal tolerance, impaired glucose tolerance, or diabetes by World Health Organization criteria. Among subjects at least 25 years old with normal tolerance, impaired glucose tolerance, or diabetes, age-sex-adjusted mortality through 1983 was, respectively,  $39 \pm 2$ ,  $49 \pm 4$ , and  $71 \pm 4$  deaths/1000 person-years ( $\pm$  standard error) for all causes ( $p < .001$  for difference in 3 groups), and  $24 \pm 2$ ,  $25 \pm 3$ , and  $40 \pm 3$ , respectively, for vascular causes (cardiovascular, cerebrovascular, or renal disease) ( $p < .001$ ). 206 men with abnormal tolerance by local, but not World Health Organization, criteria were randomly assigned to diet with tolbutamide, diet only, or no treatment, which was continued through 1975. Age-adjusted all-cause mortality through 1983 did not differ significantly among treatment groups ( $34 \pm 9$ ,  $52 \pm 10$ ,  $45 \pm 19$ ), but vascular mortality was  $10 \pm 5$ ,  $31 \pm 8$ , and  $38 \pm 19$ , respectively, in those assigned to tolbutamide, diet only, or no treatment ( $p < .05$ ). Thus compared with persons with normal tolerance, diabetic subjects had higher all-cause and vascular mortality, and those with impaired glucose tolerance had higher all-cause but similar vascular mortality. Treatment of abnormal glucose tolerance apparently reduced vascular but not total mortality.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69026-04 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Carbohydrate and Energy Metabolism in Human Muscle

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: B.L. Nyomba Visiting Associate CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Dept. of Kinesiology, Univ. of Illinois, Urbana IL (A. Katz)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

0.42

PROFESSIONAL:

0.42

OTHER:

0.00

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Studies were performed to investigate the hormonal regulation and the effects of exercise, anoxia, and fasting on the regulation of glycolysis, glucose oxidation, and energy metabolism in human skeletal muscle. In particular, investigations were focused on glucose 1,6-bisphosphate (GP2) as a possible important regulator of glycolysis, since previous studies had shown it to increase in parallel with glycolysis after isometric contraction of the muscle to fatigue. Glucose metabolites in muscle during prolonged fasting are currently being analyzed. However, GP2 increases in human skeletal muscle following either isometric contraction, anoxia, circulatory occlusion, and in response to insulin and epinephrine. There is no increase in GP2 when glucose is infused in the absence of insulin. GP2 activation may be a result of activation of GP2 synthase by its substrates glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). Thus, it appears that GP2 may have a significant role to play in stimulating glycolysis under a variety of metabolic conditions in human skeletal muscle. The ultimate mechanism involves the activation of phosphofructokinase by GP2.

This study will be terminated next year.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Insulin Receptor Tyrosine Kinase in Insulin Resistance in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: B.L. Nyomba Visiting Associate CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.12

## PROFESSIONAL:

0.12

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To investigate the possible role of the insulin receptor tyrosine kinase in the pathogenesis of insulin resistance in Pima Indians, the kinase activity was determined in muscle biopsies obtained before and during a two-step euglycemic, hyperinsulinemic clamp in diabetic and non-diabetic Pima Indians. The kinase activity increased with insulin infusion in vivo in a dose-dependent manner and its stimulation by insulin in diabetics was less than in non-diabetic subjects. However, there was no direct relationship between the kinase activity and insulin action. On the contrary, the sensitivity of the kinase to insulin was inversely related to insulin action and directly related to plasma insulin levels.

In another study, we induced changes in insulin action in non-diabetic Pima Indians and Caucasians by feeding them each of a "traditional" high carbohydrate, Pima-type diet, and a "modern," high-fat Western-type diet. We then determined the receptor kinase activity and estimated insulin action by the minimal model method. The modern diet induced insulin resistance and increased the kinase activity in the majority of subjects. The changes in kinase activity were negatively correlated with the changes in insulin action and positively correlated with the changes in plasma insulin levels.

In conclusion, the insulin receptor tyrosine kinase activity, while defective in type II diabetes, is not directly associated with insulin action in non-diabetic subjects and is not the site of in vivo insulin resistance in Pima Indians. It is possible that the kinase increases with plasma insulin levels as part of a mechanism compensating for insulin resistance at a distal site. We are currently studying the mechanisms of this compensation at the kinase level by assessing the phosphotyrosine-phosphoserine/ threonine content of the receptor. This study includes a time-course of the kinase activation by insulin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69028-02 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetics of Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	P.H. Bennett	Chief	PECRB, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK

COOPERATING UNITS (if any)

Collaborative Research, Waltham, MA, Bowman-Gray Medical School, Winston-Salem, NC

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

0.5

OTHER:

0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Non-insulin dependent diabetes mellitus is a common chronic disease that develops in most populations in late middle age. The Pima Indians of Arizona have the highest reported prevalence of this disease in the world and in contrast to many populations the disease often presents at an earlier age. As a result of long-term epidemiological studies in the total population, the familial nature of the disease has been well documented, and segregation analyses suggest the possibility of inheritance by a single co-dominant major gene.

This project will search for genetic determinants of NIDDM using the techniques of genetic linkage analysis with restriction fragment length polymorphism markers (RFLPs) to identify the chromosomal location of inherited determinants of NIDDM in the Pima Indian population. A number of informative pedigrees have been identified and lymphoblast cell lines from informative members of these pedigrees established. DNA from these lymphoblasts will be isolated and polymorphic probes will be applied to search for evidence of linkage of these markers and NIDDM. Probes with established chromosomal locations will be used to screen the genome to detect genetic linkage with NIDDM.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Skeletal Muscle Ribosomal Protein S6 Kinase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Sommercorn Senior Staff Fellow CDNS, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK  
M. DeGregorio Visiting Fellow CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.42

## PROFESSIONAL:

0.42

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We examined the influence of insulin on S6 kinase activity in skeletal muscle of 7 insulin-sensitive, 4 insulin-resistant, non-diabetic, and 5 diabetic Pima Indians. S6 kinase activity was assayed in extracts biopsies obtained at 0, 15, 30, 45, 60, and 90 minutes during a hyperinsulinemic (~2,000 U/ml plasma), euglycemic clamp. Basal S6 kinase activity was similar in the groups (~0.5 pmol/min mg protein). In sensitive subjects, S6 kinase activity increased sharply between 15 and 30 minutes of insulin infusion, reaching a maximum (4.6-fold increase) at 45 minutes, before declining. S6 kinase activity also increased (3x) in resistant subjects, but the maximum occurred at 60 minutes, without a rapid increase between 15 and 30 minutes. The timecourse of S6 kinase activation in diabetic subjects was similar to that of resistant subjects. The absence of the early response suggested that insulin-resistant subjects may lack a particular early-responding S6 kinase. However, chromatography of extracts on FPLC monoQ revealed two peaks of insulin-stimulated S6 kinase activity that eluted identically in the two groups. The majority of the increased activity occurred in peak 2. Immunoblot analysis revealed that the enzyme responsible for peak 1 activity is antigenically related to the 90 kilodalton S6 kinase and that peak 2 activity, which accounts for the majority of the stimulation of total S6 kinase activity by insulin, is accounted for by an enzyme antigenically related to the 70 kilodalton S6 kinase. The abnormal time course of S6 kinase activation in resistant and diabetic subjects is not explained by the lack of a particular S6 kinase but rather by a lower magnitude and rate of stimulation of both S6 kinase activities. These results suggest that the biochemical lesion responsible for insulin resistance in Pima Indians occurs upstream from the S6 kinases in the pathway of insulin signal transduction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69030-02 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Contribution of Protein Tyrosine Phosphatase to Insulin Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Sommercorn Senior Staff Fellow CDNS, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK  
R. Maeda Visiting Fellow CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Dept. of Biochemistry, Univ. of WA, Seattle, WA (N. Tonks)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:  
0.62

PROFESSIONAL:  
0.62

OTHER:  
0.00

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The protein tyrosine phosphatases are enzymes that specifically dephosphorylate phosphotyrosine in proteins. As such, they could attenuate the signal generated by the insulin receptor and thus contribute to insulin resistance. The objectives of these studies are 1) to determine if subjects with insulin resistance have abnormally high protein tyrosine phosphatase activity in skeletal muscle, 2) to determine if insulin regulates one or more protein tyrosine phosphatase activities in skeletal muscle, and 3) to determine if the effect of insulin on PTPase activity in skeletal muscle is altered in insulin resistance.

The influence of insulin on PTPase activities in human skeletal muscle has been examined in 10 insulin-sensitive and 7 insulin-resistant Pima Indians. Basal PTPase activity in the soluble fraction was the same in sensitive and resistant subjects. In the particulate fraction, however, basal PTPase activity was 30% higher in resistant subjects than in sensitive subjects. Soluble PTPase activity was rapidly suppressed by 25-30% in response to insulin infusion in insulin-sensitive subjects. The effect of insulin was maximal by 15 minutes of insulin infusion and persisted throughout 45 minutes of the insulin infusion. In contrast, insulin had very little effect on soluble PTPase activity in resistant subjects. The difference between sensitive and resistant subjects was particularly apparent at early time points, such as 15 minutes, where the effect in sensitive subjects was maximal and there was virtually no effect of insulin on soluble PTPase activity in resistant subjects.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69031-02 PEGR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Phosphorylase Phosphatase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	Y. Kida	Visiting Fellow	CDNS, NIDDK
	R. Maeda	Visiting Fellow	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

0.62

PROFESSIONAL:

0.32

OTHER:

0.30

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin-stimulated glycogen synthase (GS) activity of in human muscle correlates with insulin-mediated glucose disposal rates and is reduced in insulin-resistant subjects. Activation of protein phosphatase-1 (PP-1) may contribute to the mechanism by which insulin activates GS. We investigated the change of phosphorylase phosphatase (PHP) and GS activities during a 2-hour hyperinsulinemic euglycemic clamp in 12 insulin-sensit9ve (group S) and 8 insulin-resistant (group R) subjects. Muscle biopsies were obtained from quadriceps femoris muscle at times 0, 10, 20, 40, and 120 minutes of insulin infusion.

GS fractional activity was increased significantly by 10 minutes in group S, and by 20 minutes in group R but remained low compared to group S at 120 minutes. Fasting PHP activity was low for group R compared to group S, and did not increase significantly in group R until 20 minutes. In group S, PHP was significantly stimulated by 10 minutes, remaining significantly higher than in group R at all time points. The insulin-mediated changes in phosphatase activities were not decreased by 3 nM okadaic acid (OA) but were completely inhibited by 1 uM OA verifying insulin activation of a type 1 phosphatase. Subcellular fractionation demonstrated reduced fasting PP-1 activities in both the glycogen and cytosolic fractions of muscle obtained from subjects in group R compared to those in groups S.

These results suggest that activation of PP-1 could contribute to insulin-stimulation of GS in human muscle. Lower fasting PP-1 in cytosol and glycogen subcellular fraction plus low insulin stimulated PP-1 activity could explain, in part, reduced insulin stimulated GS in insulin-resistant subjects.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Skeletal Muscle Casein Kinase II by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Sommercorn Senior Staff Fellow CDNS, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK  
R. Maeda Visiting Fellow CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

1.02

## PROFESSIONAL:

1.02

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have investigated the influence of insulin on the activity of casein kinase II (CKII) in skeletal muscle of 7 insulin-sensitive, 4 insulin-resistant, non-diabetic, and 5 diabetic Pima Indians during a 2-hour hyperinsulinemic, euglycemic clamp. In sensitive subjects, CKII was transiently activated (42%) reaching a maximum over basal activity at 45 minutes before declining. CKII was also stimulated in resistant subjects (19%) and diabetics (34%), but the activity remained elevated throughout the clamp. The magnitude of activation of CKII by insulin in muscle of resistant subjects may have been limited by the fact that basal CKII activity in this group was 40% higher than in either sensitive or diabetic subjects. The higher basal CKII activity in resistant subjects was not explained by a higher concentration of CKII protein as determined by Western blotting. Among the three groups, basal CKII activity was correlated with fasting plasma insulin concentrations suggesting that the higher basal activity in resistant subjects resulted from their higher plasma insulin concentrations. Extracts of muscle obtained from all three groups either before or after insulin administration were treated with immobilized alkaline phosphatase, which reduced and equalized CKII activity in the extracts. These results suggest that insulin stimulates CKII activity in human skeletal muscle by a mechanism involving phosphorylation of either CKII or of an effector molecule and support the idea that elevated basal activity in resistant subjects results from insulin action. Our studies suggest that the ability of insulin to activate CKII in skeletal muscle is not impaired in insulin-resistant Pima Indians, and that the biochemical lesion responsible for insulin resistance occurs either downstream from CKII or in a different pathway of insulin action.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Relationship between insulin resistance &amp; blood pressure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK
	S. Lillioja	Visiting Scientist with Tenure	CDNS, NIDDK
	B.L. Nyomba	Visiting Associate	CDNS, NIDDK
	R. Ferraro	Staff Fellow	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis and Epidemiology Section (DAES)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.45

## PROFESSIONAL:

0.45

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It has been recently proposed that insulin is the link between obesity, hypertension, and glucose intolerance. Both obesity and glucose intolerance are associated with increased insulin resistance, hyperinsulinemia, and increased prevalence of hypertension. In addition, patients with essential hypertension are insulin-resistant and hyperinsulinemic. Therefore, it has been postulated that insulin might play a role in the pathogenesis of hypertension through stimulation of the sympathetic nervous system, promoting renal sodium retention, or affecting cation transport. However, in a cross-sectional study of 2873 Pima Indians seen at the NIH research clinic, there was no relationship between hypertension or blood pressure and serum insulin concentrations. To explore this issue further, the relationship between both insulin resistance and insulin concentrations and blood pressure is being studied in Pima Indians compared to other ethnic groups (Caucasians and Blacks).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69034-02 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The long Q-T interval syndrome in diabetes mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis and Epidemiology Section (DAES)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

0.11

PROFESSIONAL:

0.11

OTHER:

0.00

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent studies indicated that diabetic autonomic neuropathy can lead to the long Q-T interval syndrome. This may increase the susceptibility of patients with diabetic autonomic neuropathy to ventricular arrhythmias and subsequent sudden death. The prevalence of and the risk factors for this condition will be determined in diabetic and non-diabetic Pima Indians by reviewing the electrocardiograms and the records of 1,000 adults seen at the NIH research clinic. In addition, 50 diabetic subjects with long Q-T interval, 50 diabetic subjects with normal Q-T interval, and 50 non-diabetic subjects with normal electrocardiograms will be studied in detail. All subjects will undergo detailed evaluation of the autonomic nervous system using a battery of standardized tests. Ambulatory cardiac monitoring will be obtained to determine the frequency of silent ischemic episodes and ventricular premature beats.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Autonomic nervous system activity in obesity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK
	E. Ravussin	Visiting Scientist	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis and Epidemiology Section (DAES)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.26

## PROFESSIONAL:

0.26

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The Pima Indians have a high prevalence of obesity with more than 70% of the adult population having a body mass index 27 kg/m<sup>2</sup>. Alteration in autonomic nervous system activity may contribute to the pathogenesis of obesity in this population. Studies in animal models of spontaneous obesity demonstrated decreased sympathetic nervous system activity. A recent study in obese humans showed decreased sympathetic and parasympathetic activity. The authors postulated that a disordered homeostatic mechanism promotes an excessive storage of energy by decreasing sympathetic activity. The relationship between obesity and autonomic nervous system activity will be studied in 50 Pima Indians aged 20-40 years. Fifty Caucasians of comparable age and weight will be included for comparison.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69036-01 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Epidemiology of Complications of Non-Insulin-Dependent Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

Others: P.H. Bennett Chief PECRB, NIDDK  
D.J. Pettitt Assistant Chief DAES, NIDDK  
M.A. Charles Special Volunteer DAES, NIDDK  
M.F. Saad Visiting Associate DAES, NIDDK  
Liu Q.Z. Visiting Fellow DAES, NIDDK  
W.J. Butler Computer Systems Analyst BDMS, NIDDK

COOPERATING UNITS (if any)

Biostatistics and Data Management Section, PECRB, NIDDK; Indian Health Service; State University of New York at Buffalo; Cleveland Clinic Foundation, Cleveland, OH.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

2.6

PROFESSIONAL:

2.0

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of the project is to determine the incidence rates, rates of progression, and risk factors for the chronic complications of NIDDM. The study is conducted in the Pima Indians of the Gila River Indian Community, who have participated in a longitudinal epidemiologic study for 25 years.

Risk factors for the major complications of diabetes, retinopathy, nephropathy, coronary artery disease, and peripheral vascular disease are determined by longitudinal followup of diabetic subjects. Methods of ascertainment of these complications include fundus photography, measurement of urine albumin and serum creatinine concentrations, electrocardiography, and documentation of lower extremity amputations.

These complications are responsible for the major morbidity and excessive mortality associated with diabetes in this population. The incidence rates of severe complications such as end stage renal disease, cataract requiring surgical treatment, and lower extremity amputations are as high among diabetic Pimas as reported anywhere else in the world. Diabetic nephropathy is the leading cause of deaths among diabetic Pimas. Late stages of nephropathy can be predicted by minor abnormalities of urinary albumin excretion early in the course of the disease and show strong familial aggregation suggesting a genetic susceptibility to nephropathy as well as to diabetes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Kidney Function in Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett

Chief

PECRB, NIDDK

Others: W.C. Knowler

Chief

DAES, NIDDK

## COOPERATING UNITS (if any)

Cleveland Clinic Foundation; Stanford University; Emory University; Chronic Renal Diseases Program, NIDDK

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.2

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The functional characteristics of the renal glomerulus are being investigated in Pima Indians of the Gila River Indian Community to identify the underlying pathogenetic mechanisms involved in the initiation and progression of renal disease in non-insulin-dependent diabetes mellitus (NIDDM).

The Pima Indian population has a high incidence of NIDDM and diabetic nephropathy. Six groups of subjects are being studied: subjects with normal glucose tolerance, individuals with impaired glucose tolerance (IGT), those with newly diagnosed diabetes (<3 years duration NIDDM), and diabetic subjects (≥5 years duration NIDDM) with evidence of (a.) mild abnormal albumin excretion (b.) severe abnormalities of albumin excretion (c.) normal albumin excretion. Measurements of renal and glomerular capillary wall function including glomerular filtration rate (GFR), renal plasma flow (RPF), dextran sieving coefficients, and albuminuria are being performed and correlated. A search for markers and/or predictors of progression as well as an attempt to uncover the mechanisms of initiation and progression of diabetic renal disease is being made.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69038-01 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin and Hypertension in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

Others: M.F. Saad Visiting Associate DAES, NIDDK  
D.J. Pettitt Assistant Chief DAES, NIDDK  
P.H. Bennett Chief PECRB, NIDDK

COOPERATING UNITS (if any)

Clinical Diabetes and Nutrition Section, PECRB, NIDDK

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.2

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It has been proposed recently that insulin is the link between obesity, hypertension, and glucose intolerance. Both obesity and glucose intolerance are associated with increased insulin resistance, hyperinsulinemia, and increased prevalence of hypertension. In addition, patients with essential hypertension are insulin resistant and hyperinsulinemic. Therefore, it has been postulated that insulin might play a role in the pathogenesis of hypertension through stimulation of the sympathetic nervous system, promoting renal sodium retention, or affecting cation transport. The relationships between insulinemia, obesity, glucose intolerance and hypertension have been studied in 2873 Pimas.

Among subjects not taking antihypertensive drugs, neither fasting nor post-load serum insulin concentrations were related to blood pressure. Thus these findings do not support the hypothesis of a role for insulin in determining hypertension or regulating blood pressure in the Pima Indians.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69039-01 PECR

PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dietary Survey of the Pima Indians of the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

Others: P.H. Bennett Chief PECRB, NIDDK  
D.J. Pettitt Assistant Chief DAES, NIDDK

COOPERATING UNITS (if any)

Cleveland Clinic Foundation, Phoenix, Arizona.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An age-sex-stratified sample of 600 residents of the Gila River Indian Community, ages 18-75 years, was recruited for a dietary survey. Dietary intake was estimated by the dietary history method to obtain quantitative food frequency information. Reported energy intake was higher in men and negatively related to age; thus, relationships with weight were analyzed by multiple regression controlling for sex and age. Energy intake in kilocalories (kcal) was positively associated with body weight or body mass index, adjusted for age and sex. Body weight was associated with absolute intake of the major dietary components, carbohydrate, fat, and protein, but not with any of these components expressed per 1000 kcal, suggesting that total energy intake, rather than proportions of specific components, was the variable having the strongest association with body weight. Alcohol consumption (in any amount) was higher in men and inversely associated with age and weight. Neither energy intake nor specific components were significantly associated with diabetes, after adjustment for age and sex, nor did diabetes affect the relationship between energy and weight, in a subset of 307 subjects (153 diabetics) examined for diabetes within one year of the diet history. The acceptance of obesity in this population may reduce the under-reporting of energy intake which has been postulated in other studies. Body weight in Pima Indians is weakly but positively associated with increased caloric intake.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sodium-Lithium Countertransport and Blood Pressure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

Others: M.F. Saad Visiting Associate DAES, NIDDK  
D.J. Pettitt Assistant Chief DAES, NIDDK

## COOPERATING UNITS (if any)

University of Pittsburgh.

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.2

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Red blood cell sodium lithium countertransport is a genetic marker of hypertension in several ethnic groups. It reflects the sodium-hydrogen antiport activity in renal tubules. Several recent studies showed an association between sodium-lithium countertransport and predisposition to diabetic nephropathy. We are studying the relationships between sodium-lithium countertransport, nephropathy, and blood pressure in a sample of 200 Pima Indians.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance in Obesity and the Association with Lymph Insulin Kinetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist with tenure	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.32

## PROFESSIONAL:

0.32

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

- |  |  |                                      |
|--|--|--------------------------------------|
| <input checked="" type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors                   |  |                                      |
| <input type="checkbox"/> (a2) Interviews               |  |                                      |

## SUMMARY OF WORK (Use standard unraduced type. Do not exceed the space provided.)

We have previously shown that the density of capillary supply in skeletal muscle of man correlates with insulin resistance. We postulated that since the unfenestrated capillaries of muscle are relatively impermeable to insulin then the increased insulin resistance in those with low capillary density might be due to altered kinetics of insulin penetration to its sites of action in muscle. To test this hypothesis further we have developed a method of directly collecting lymph from a peripheral lymphatic. The method allows the collection of 0.7 to 3.7 ml/hour of lymph which is sufficient for analysis for glucose, insulin, and labeled inulin. Preliminary results have shown that insulin concentrations in lymph rise very slowly after plasma insulin levels are raised and by 2-1/2 hours are still only about 30% of arterial concentrations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of nicotinic acid-induced insulin resistance on B-cell function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus

Chief

CDNS, NIDDK

Others: M.F. Saad

Visiting Associate

DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis and Epidemiology Section (DAES)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.12

## PROFESSIONAL:

0.12

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Both insulin resistance and beta-cell dysfunction contribute to the pathogenesis of non-insulin dependent diabetes mellitus but controversy exists regarding which lesion is primary. In this study insulin resistance will be induced by administration of nicotinic acid (2 g orally daily for 4 weeks) in 15 lean (body mass index <25 kg/m<sup>2</sup>) Caucasian males with no family history of non-insulin dependent diabetes. The changes in glucose tolerance and beta-cell function will be studied.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69043-01 PECR

## PERIOD COVERED

October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of gene expression by insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Sommercorn Senior Staff Fellow CDNS, NIDDK

Others: D.B. Thompson IRTA CDNS, NIDDK  
M. DeGregorio Visiting Fellow CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.50

## PROFESSIONAL:

0.50

## OTHER:

0.00

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin resistance, which precedes the development of non-insulin dependent diabetes mellitus in Pima Indians, appears to result from a postreceptor defect in signal transduction that affects the ability of insulin to regulate glycogen synthase activity in skeletal muscle. There is evidence in the literature that the influences of insulin on gene transcription may be mediated by a mechanism different from that by which the hormone influences metabolic processes as much as glycogen synthesis. Accordingly, we are undertaking this project to determine if insulin resistance in Pima Indians affects the ability of insulin to regulate transcription of the insulin-sensitive glucose transporter gene (GLUT 4) and of the proto-oncogene, c-fos. The project will also involve identification of insulin-sensitive cis-acting elements in the promoter regions of these genes and characterization of putative trans-acting factors, that may bind to these elements and mediate effects of insulin on transcription.









NIH Library, Building 10, 10th  
National Institutes of Health  
Bethesda, Md. 20892-0001



Amazing Research.  
Amazing Help.

<http://nihlibrary.nih.gov>

---

10 Center Drive  
Bethesda, MD 20892-1150  
301-496-1080



